PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 25 August 1999 (25.08.99)	
International application No. PCT/DE98/03817	**Applicant's or agent's file reference** 180-1 PCT
International filing date (day/month/year) 18 December 1998 (18.12.98)	**Priority date** (day/month/year) 19 December 1997 (19.12.97)
Applicant BRÜSTLE, Oliver	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

07 July 1999 (07.07.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer R. Forax
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992) 2809003

PCT

WELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 6 :
C12N 5/08, 5/10, A61K 35/30, 48/00

A3

(11) Internationale Veröffentlichungsnummer: WO 99/32606

(43) Internationales Veröffentlichungsdatum: 1. Juli 1999 (01.07.99)

(21) Internationales Aktenzeichen: PCT/DE98/03817

(22) Internationales Anmeldedatum: 18. Dezember 1998 (18.12.98)

(30) Prioritätsdaten:
197 56 864.5 19. Dezember 1997 (19.12.97) DE

(71)(72) Anmelder und Erfinder: BRÜSTLE, Oliver [DE/DE]. Lindenweg 17, D-53340 Meckenheim (DE).

(74) Anwälte: VOSSIUS, Volker usw.; Holbeinstrasse 5, D-81679 München (DE).

(81) Bestimmungsstaaten: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht
Mit internationalem Recherchenbericht.

(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 26. August 1999 (26.08.99)

(54) Title: NEURAL PRECURSOR CELLS, METHOD FOR THE PRODUCTION AND USE THEREOF IN NEURAL DEFECT THERAPY

(54) Bezeichnung: NEURALE VORLÄUFERZELLEN, VERFAHREN ZU IHRER HERSTELLUNG UND IHRE VERWENDUNG ZUR THERAPIE VON NEURALEN DEFEKTEN

(57) Abstract

The invention relates to isolated and purified neural precursor cells, to a method for the production thereof from embryonal stem cells in unlimited quantities, to the use of neural precursor cells in neural defect therapy, especially in mammals, preferably human beings, and to obtain polypeptides.

(57) Zusammenfassung

Die Erfindung betrifft isolierte und gereinigte neurale Vorläuferzellen, Verfahren zu ihrer Herstellung aus embryonalen Stammzellen in unbegrenzter Menge, die Verwendung der neuralen Vorläuferzellen zur Therapie von neuralen Defekten insbesondere bei Säugern, vorzugsweise Menschen und zur Gewinnung von Polypeptiden.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AL	Albanien	ES	Spanien	LS	Lesotho	SI	Slowenien
AM	Armenien	FI	Finnland	LT	Litauen	SK	Slowakei
AT	Österreich	FR	Frankreich	LU	Luxemburg	SN	Senegal
AU	Australien	GA	Gabun	LV	Lettland	SZ	Swasiland
AZ	Aserbaidschan	GB	Vereinigtes Königreich	MC	Monaco	TD	Tschad
BA	Bosnien-Herzegowina	GE	Georgien	MD	Republik Moldau	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagaskar	TJ	Tadschikistan
BE	Belgien	GN	Guinea	MK	Die ehemalige jugoslawische	TM	Turkmenistan
BF	Burkina-Faso	GR	Griechenland		Republik Mazedonien	TR	Türkei
BG	Bulgarien	HU	Ungarn	ML	Mali	TT	Trinidad und Tobago
BJ	Benin	IE	Irland	MN	Mongolei	UA	Ukraine
BR	Brasilien	IL	Israel	MR	Mauretanien	UG	Uganda
BY	Belarus	IS	Island	MW	Malawi	US	Vereinigte Staaten von
CA	Kanada	IT	Italien	MX	Mexiko		Amerika
CF	Zentralafrikanische Republik	JP	Japan	NE	Niger	UZ	Usbekistan
CG	Kongo	KE	Kenia	NL	Niederlande	VN	Vietnam
CH	Schweiz	KG	Kirgisistan	NO	Norwegen	YU	Jugoslawien
CI	Côte d'Ivoire	KP	Demokratische Volksrepublik	NZ	Neuseeland	ZW	Zimbabwe
CM	Kamerun		Korea	PL	Polen		
CN	China	KR	Republik Korea	PT	Portugal		
CU	Kuba	KZ	Kasachstan	RO	Rumänien		
CZ	Tschechische Republik	LC	St. Lucia	RU	Russische Föderation		
DE	Deutschland	LI	Liechtenstein	SD	Sudan		
DK	Dänemark	LK	Sri Lanka	SE	Schweden		
EE	Estland	LR	Liberia	SG	Singapur		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DE 98/03817

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08 C12N5/10 A61K35/30 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 02049 A (EMORY UNIVERSITY) 23 January 1997 see the whole document ---	1-3,18, 23,31-38
A	WO 97 16534 A (GENENTECH) 9 May 1997 see the whole document ---	1-38
A	WO 95 12665 A (DIACRIN, INC.) 11 May 1995 see the whole document ---	1-38
A	OKABE S ET AL: "Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro." MECHANISMS OF DEVELOPMENT, (1996 SEP) 59 (1) 89-102, XP002105103 cited in the application see the whole document --- -/--	1-38

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 June 1999	21/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DE 98/03817

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	BRUSTLE O ET AL: "In vitro-generated neural precursors participate in mammalian brain development." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 DEC 23) 94 (26) 14809-14, XP002105104 see the whole document	1,18,23, 31-38

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DE 98/03817

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Oberservation: Although Claims Nos. 31-36 relate to a method for treatment of the human or animal body, the search was carried out and was based on the cited effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DE 98/03817

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9702049 A	23-01-1997	US 5753505 A	19-05-1998
		AU 6452196 A	05-02-1997
		CA 2226417 A	23-01-1997
		EP 0841950 A	20-05-1998
WO 9716534 A	09-05-1997	AU 704976 B	13-05-1999
		AU 7663496 A	22-05-1997
		CA 2234230 A	09-05-1997
		EP 0858502 A	19-08-1998
WO 9512665 A	11-05-1995	EP 0725817 A	14-08-1996

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/DE 98/03817

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 6 C12N5/08 C12N5/10 A61K35/30 A61K48/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 6 C12N A61K

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	WO 97 02049 A (EMORY UNIVERSITY) 23. Januar 1997 siehe das ganze Dokument ---	1-3,18, 23,31-38
A	WO 97 16534 A (GENENTECH) 9. Mai 1997 siehe das ganze Dokument ---	1-38
A	WO 95 12665 A (DIACRIN, INC.) 11. Mai 1995 siehe das ganze Dokument ---	1-38
A	OKABE S ET AL: "Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro." MECHANISMS OF DEVELOPMENT, (1996 SEP) 59 (1) 89-102, XP002105103 in der Anmeldung erwähnt siehe das ganze Dokument --- -/--	1-38

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

° Besondere Kategorien von angegebenen Veröffentlichungen :
"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist
"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist
"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)
"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht
"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist
"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist
"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden
"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist
"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
7. Juni 1999	21/06/1999

Name und Postanschrift der Internationalen Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr
P,X	BRUSTLE O ET AL: "In vitro-generated neural precursors participate in mammalian brain development." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 DEC 23) 94 (26) 14809-14, XP002105104 siehe das ganze Dokument	1,18,23, 31-38

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen

PCT/DE 98/03817

Feld I Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 2 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. [X] Ansprüche Nr.
weil sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich

Bemerkung: Obwohl die Ansprüche 31-36 sich auf ein Verfahren zur Behandlung des menschlichen/tierischen Körpers beziehen, wurde die Recherche durchgeführt und gründete sich auf die angeführten Wirkungen der Verbindung/Zusammensetzung.

2. [ ] Ansprüche Nr.
weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich

3. [ ] Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 3 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. [ ] Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche.

2. [ ] Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Behörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

3. [ ] Da der Anmelder nur einige der erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht nur auf die Ansprüche, für die Gebühren entrichtet worden sind, nämlich auf die Ansprüche Nr.

4. [ ] Der Anmelder hat die erforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen erfaßt:

Bemerkungen hinsichtlich eines Widerspruchs

[ ] Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt.

[ ] Die Zahlung zusätzlicher Recherchengebühren erfolgte ohne Widerspruch.

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Internationales Aktenzeichen
PCT/DE 98/03817

Im Recherchenbericht angeführtes Patentdokument		Datum der Veröffentlichung	Mitglied(er) der Patentfamilie			Datum der Veröffentlichung
WO 9702049	A	23-01-1997	US	5753505	A	19-05-1998
			AU	6452196	A	05-02-1997
			CA	2226417	A	23-01-1997
			EP	0841950	A	20-05-1998
WO 9716534	A	09-05-1997	AU	704976	B	13-05-1999
			AU	7663496	A	22-05-1997
			CA	2234230	A	09-05-1997
			EP	0858502	A	19-08-1998
WO 9512665	A	11-05-1995	EP	0725817	A	14-08-1996

Translation

PATENT COOPERATION TREA

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 180-1 PCT	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/DE98/03817	International filing date (*day/month/year*) 18 December 1998 (18.12.98)	Priority date (*day/month/year*) 19 December 1997 (19.12.97)
International Patent Classification (IPC) or national classification and IPC C12N 5/08		
Applicant BRÜSTLE, Oliver		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07 July 1999 (07.07.99)	21 March 2000 (21.03.2000)
Name and mailing address of the IPEA/EP Facsimile No.	Authorized officer Telephone No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/DE98/03817

I. Basis of the report

1. This report has been drawn on the basis of (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

☐ the international application as originally filed.

☒ the description, pages 1-40, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____.

☒ the claims, Nos. 1-38, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. _____, filed with the letter of _____,
Nos. _____, filed with the letter of _____.

☒ the drawings, sheets/fig 1/8-8/8, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☐ the description, pages _____
☐ the claims, Nos. _____
☐ the drawings, sheets/fig _____

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-38	YES
	Claims		NO
Inventive step (IS)	Claims		YES
	Claims	1-38	NO
Industrial applicability (IA)	Claims	1-30	YES
	Claims	31-38	NO

2. Citations and explanations

Reference is made to the following documents:

D1: WO-A-97/02049 (EMORY UNIVERSITY), 23 January 1997

D2: OKABE S. ET AL.: MECHANISMS OF DEVELOPMENT, (1996 SEP)59(1)89-102

D3: WO-A-95/12665 (Diacrin Inc.), 11 May 1995

The report of 20 January 2000.

1. Novelty (PCT Article 33(2))

1.1 Document D1, which is considered the closest prior art, discloses isolated, non tumour-derived, purified progenitor cells with neuronal properties derived from the cells of the anterior subventricular zone of a mammalian brain (see the abstract and page 7).

The isolated cellular composition described in D1 contains more than 90% of these mammalian neuronal progenitor cells, which express a nerve-specific marker and are capable of developing into cells which differentiate into neuronal cells. D1 further discloses a method for developing this isolated

cellular composition of neuronal progenitor cells (see page 6), and also methods for treating diseases of the nervous system using these progenitor cells (see pages 4 and 5).

1.2 The subject matter of Claim 1 of the present application differs from the neuronal progenitor cells known from D1 in that the cells of Claim 1 originate from embryonic stem cells.

The subject matter of Claim 1 is therefore novel (PCT Article 33(2)). The same applies to Claims 2-17, which are dependent on Claim 1, and to Claims 18-38, which concern methods for producing these progenitor cells or the use of these cells.

2. Inventive step (PCT Article 33(3))

2.1 D1 is considered the closest prior art.

As explained in point 1.2, D1 does not concern neuronal progenitor cells extracted from embryonic stem cells.

As a result of this distinguishing feature, no brain tissue, which is mainly extracted from embryonic donor brains, is required as starting material.

The problem addressed by the invention can therefore be considered to be that of providing an alternative source of neuronal progenitor cells having the same advantages as the cells known from D1.

2.2 A person skilled in the art seeking to solve this problem would also consider D2, which deals with the

same problem (see also page 89). That document investigates the development *in vitro* of neuronal progenitor cells from embryonic stem cells.

After bFGF (basic fibroblast growth factor) is removed, highly purified neuroepithelial progenitor cells are obtained from the embryonic stem cells. After further cell differentiation in a serum-containing medium, the neurons express a wide variety of nerve-specific genes (see the abstract).

It is acknowledged that D2 does not mention the use of such cells for transplantation, nor that those cells achieve such a high degree of purity.

However, as suggested in the application itself and in the letter of 20 January 2000, the use of growth factor *combinations* and the type of cell culture (temporarily as spheroids; Claim 23) are necessary in order to achieve the degree of purity of the progenitor cells that distinguishes these cells from the prior art, or that makes them suitable for transplantation purposes.

2.3 It therefore appears obvious for a person skilled in the art to use the embryonic stem cells described in D2 for developing the known progenitor cells with the same advantageous properties, thus arriving at the subject matter of Claim 1.

Consequently, the subject matter of Claim 1 does not appear to involve the inventive step required by PCT Article 33(3).

The same arguments also apply to the subject matter

of independent Claims 2, 4, 18 and 23, which deal with a process for producing said progenitor cells.

An inventive step is not recognised in dependent Claims 2, 3, 5-17, 19-22 and 24-30 either, since the additional characterising features of these claims are essentially known from D1 and/or D2.

D3, which concerns the differentiation of embryonic stem cells in desired cell lines, such as neuronal cell lines, also describes said properties of dependent Claims 2, 3, 5-17, 19-22 and 24-30.

The characterising features of Claims 2, 3, 5-17, 19-22 and 24-30 which are not known from the above-mentioned documents obviously represent only simple technical features that lie within the scope of normal trade practice and knowledge, or obvious modifications.

Claims 31-38 do not meet the requirements of PCT Article 33(3) either, because the subject matter of these claims concerns obvious possibilities for using such progenitor cells that cannot be regarded as inventive.

2.4 However, it is recognised that an inventive step could be convincingly demonstrated by including in the claims the characteristic features (growth factor *combinations*, AND the type of cell culture via spheroids) that lead to neuronal progenitor cells that differ from the prior art.

3. Industrial applicability

3.1 Claims 31-36 concern a subject matter which, in the opinion of the Examining Authority, falls under PCT Rule 67.1(iv). Consequently, no expert opinion is given on the industrial applicability of the subject matter of these claims (PCT Article 34(4)(a)(i)).

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

PCT

INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

Aktenzeichen des Anmelders oder Anwalts	WEITERES VORGEHEN	siehe Mitteilung über die Übermittlung des internationalen Recherchenberichts (Formblatt PCT/ISA/220) sowie, soweit zutreffend, nachstehender Punkt 5
180-1 PCT		
Internationales Aktenzeichen	Internationales Anmeldedatum *(Tag/Monat/Jahr)*	(Frühestes) Prioritätsdatum *(Tag/Monat/Jahr)*
PCT/DE 98/03817	18/12/1998	19/12/1997
Anmelder		
BRÜSTLE, Oliver		

Dieser internationale Recherchenbericht wurde von der Internationalen Recherchenbehörde erstellt und wird dem Anmelder gemäß Artikel 18 übermittelt. Eine Kopie wird dem Internationalen Büro übermittelt.

Dieser internationale Recherchenbericht umfaßt insgesamt 4 Blätter.

[X] Darüber hinaus liegt ihm jeweils eine Kopie der in diesem Bericht genannten Unterlagen zum Stand der Technik bei.

1. **Grundlage des Berichts**

   a. Hinsichtlich der **Sprache** ist die internationale Recherche auf der Grundlage der internationalen Anmeldung in der Sprache durchgeführt worden, in der sie eingereicht wurde, sofern unter diesem Punkt nichts anderes angegeben ist.

   [ ] Die internationale Recherche ist auf der Grundlage einer bei der Behörde eingereichten Übersetzung der internationalen Anmeldung (Regel 23.1 b)) durchgeführt worden.

   b. Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** ist die internationale Recherche auf der Grundlage des Sequenzprotokolls durchgeführt worden, das

   [ ] in der internationalen Anmeldung in Schriftlicher Form enthalten ist.

   [ ] zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.

   [ ] bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.

   [ ] bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.

   [ ] Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.

   [ ] Die Erklärung, daß die in computerlesbarer Form erfaßten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

2. [X] **Bestimmte Ansprüche haben sich als nicht recherchierbar erwiesen** (siehe Feld I).

3. [ ] **Mangelnde Einheitlichkeit der Erfindung** (siehe Feld II).

4. Hinsichtlich der **Bezeichnung der Erfindung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut von der Behörde wie folgt festgesetzt:

5. Hinsichtlich der **Zusammenfassung**

   [X] wird der vom Anmelder eingereichte Wortlaut genehmigt.

   [ ] wurde der Wortlaut nach Regel 38.2b) in der in Feld III angegebenen Fassung von der Behörde festgesetzt. Der Anmelder kann der Behörde innerhalb eines Monats nach dem Datum der Absendung dieses internationalen Recherchenberichts eine Stellungnahme vorlegen.

6. Folgende Abbildung der **Zeichnungen** ist mit der Zusammenfassung zu veröffentlichen: Abb. Nr. _____

   [ ] wie vom Anmelder vorgeschlagen

   [X] keine der Abb.

   [ ] weil der Anmelder selbst keine Abbildung vorgeschlagen hat.

   [ ] weil diese Abbildung die Erfindung besser kennzeichnet.

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/DE 98/03817

Feld I Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 2 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. [X] Ansprüche Nr.
weil sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich

Bemerkung: Obwohl die Ansprüche 31-36 sich auf ein Verfahren zur Behandlung des menschlichen/tierischen Körpers beziehen, wurde die Recherche durchgeführt und gründete sich auf die angeführten Wirkungen der Verbindung/Zusammensetzung.

2. [ ] Ansprüche Nr.
weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich

3. [ ] Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 3 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. [ ] Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche.

2. [ ] Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Behörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

3. [ ] Da der Anmelder nur einige der erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht nur auf die Ansprüche, für die Gebühren entrichtet worden sind, nämlich auf die Ansprüche Nr.

4. [ ] Der Anmelder hat die erforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen erfaßt:

Bemerkungen hinsichtlich eines Widerspruchs

[ ] Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt.

[ ] Die Zahlung zusätzlicher Recherchengebühren erfolgte ohne Widerspruch.

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/DE 98/03817

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 6 C12N5/08 C12N5/10 A61K35/30 A61K48/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 6 C12N A61K

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	WO 97 02049 A (EMORY UNIVERSITY) 23. Januar 1997 siehe das ganze Dokument ---	1-3,18, 23,31-38
A	WO 97 16534 A (GENENTECH) 9. Mai 1997 siehe das ganze Dokument ---	1-38
A	WO 95 12665 A (DIACRIN, INC.) 11. Mai 1995 siehe das ganze Dokument ---	1-38
A	OKABE S ET AL: "Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro." MECHANISMS OF DEVELOPMENT, (1996 SEP) 59 (1) 89-102, XP002105103 in der Anmeldung erwähnt siehe das ganze Dokument --- -/--	1-38

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

° Besondere Kategorien von angegebenen Veröffentlichungen :

"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden

"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
7. Juni 1999	21/06/1999

Name und Postanschrift der Internationalen Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

1

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
P,X	BRUSTLE O ET AL: "In vitro-generated neural precursors participate in mammalian brain development." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 DEC 23) 94 (26) 14809-14, XP002105104 siehe das ganze Dokument	1,18,23, 31-38

INTERNATIONAL SEARCH REPORT

nation on patent family members

ernational Application No
PCT/DE 98/03817

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9702049 A	23-01-1997	US 5753505 A	19-05-1998
		AU 6452196 A	05-02-1997
		CA 2226417 A	23-01-1997
		EP 0841950 A	20-05-1998
WO 9716534 A	09-05-1997	AU 704976 B	13-05-1999
		AU 7663496 A	22-05-1997
		CA 2234230 A	09-05-1997
		EP 0858502 A	19-08-1998
WO 9512665 A	11-05-1995	EP 0725817 A	14-08-1996

ELSEVIER

Mechanisms of Development 59 (1996) 89–102

Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro

Shigeo Okabe[a], Karin Forsberg-Nilsson[a,1], A. Cyril Spiro[a,1], Menahem Segal[b], Ronald D.G. McKay[a,*]

[a]*Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, 20892 MD, USA*
[b]*Department of Neurobiology, The Weizmann Institute of Science, Rehovot, 76100, Israel*

Received 13 May 1996; revision received 18 June 1996; accepted 20 June 1996

Abstract

To understand the mechanism of the sequential restriction of multipotency of stem cells during development, we have established culture conditions that allow the differentiation of neuroepithelial precursor cells from embryonic stem (ES) cells. A highly enriched population of neuroepithelial precursor cells derived from ES cells proliferates in the presence of basic fibroblast growth factor (bFGF). These cells differentiate into both neurons and glia following withdrawal of bFGF. By further differentiating the cells in serum-containing medium, the neurons express a wide variety of neuron-specific genes and generate both excitatory and inhibitory synaptic connections. The expression pattern of position-specific neural markers suggests the presence of a variety of central nervous system (CNS) neuronal cell types. These findings indicate that neuronal precursor cells can be isolated from ES cells and that these cells can efficiently differentiate into functional post-mitotic neurons of diverse CNS structures.

Keywords: Embryonic stem cell; Central nervous system; Neuronal precursor cell; In vitro culture; Synapse

1. Introduction

Early in the central nervous system (CNS) development, most of the cells which make up the neuroectoderm take the shape of columnar epithelium (neuroepithelium) and proliferate rapidly. Later in development, these cells stop dividing and differentiate into either neurons, astrocytes, or oligodendrocytes on a precise schedule. Although the proliferative properties of these neuroepithelial precursor cells and their subsequent differentiation can be effectively studied by primary culture of the neuroepithelial tissue (Gensburger et al., 1987; Caltaneo and McKay, 1990; Collazo et al., 1992; Ghosh and Greenberg, 1995; Vicario-Abejon et al., 1995), the early development of the neuroectoderm lineage is not accessible. In this sense, establishment of a system which generates neuroectodermal lineage cells from early pluripotent cells in vitro will provide a powerful tool to dissect the molecular events which regulate determination of these lineages during early embryonic development.

Mouse embryonic stem (ES) cells are pluripotent cell lines and their developmental state is equivalent to cells of the inner cell mass (ICM) in the blastocyst stage embryo (Martin, 1981). Previous studies have shown that ES cells can differentiate toward different lineages in vitro (Doetschman et al., 1985). If the in vitro differentiation process of ES cells follows the development of neuroectodermal lineages in vivo, we can expect to generate neuroepithelial precursor cells which can further differentiate into both neuronal and glial cell lineages. Extensive studies in vitro and in vivo show that the intermediate filament protein nestin is expressed in precursors to neurons, astrocytes and oligodendrocytes (Lendahl et al., 1990). In this paper we report the characterization of a nestin-positive proliferating cell population derived from ES cells in serum-free conditions. These cells require basic fibroblast growth factor (bFGF) for their proliferation and differentiate into neuronal and glial cells by withdrawal of the mitogen. After further differentiation in the presence of serum, neuronal cells make functional excitatory and inhibitory synapses and *N*-methyl-D-

* Corresponding author. NIH/NINDS/LMB, Bldg 36, Rm 3D02, 36 Convent Drive, MSC 4092, Bethesda, MD 20892-4092, USA. Tel.: +1 301 4966574; fax: +1 301 4024738.

[1] These two authors contributed equally to this work.

PII S0925-4773(96)00572-2

aspartate (NMDA) responses. These observations support the hypothesis that the differentiation pathway from pluripotential ICM cells to fully differentiated postmitotic neurons and glial cells can be dissected into intermediate steps and effectively analyzed by using the in vitro differentiation system of ES cells.

2. Results

2.1. Differentiation of neuronal precursor cells from ES cells

Undifferentiated ES cells were aggregated and cultured as a suspension for 4 days. This aggregate (embryoid body; EB) was allowed to spread out onto a permissive substrate. On the day after re-attachment of EBs, the medium was replaced with Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with insulin, transferrin, selenium and fibronectin (ITSFn medium), which was previously shown to be effective for neuronal induction in embryonal carcinoma cell lines (Rizzino and Growley, 1980). At this time point, there were no neural lineage cells in culture, confirmed by immunostaining with several antibodies specific to the early neural lineage (data not shown). During the first 72 h, a proportion of the cells detached from the plate and lysed. The remaining cells changed their morphology from tightly-packed epithelial cells to small elongated cells. By 5–7 days in ITSFn medium, a large proportion of the surviving cells developed this small elongated shape. Fig. 1A shows a phase contrast image of the morphology of these cells derived from the J1 ES cell line. Similar results were obtained with other cell lines (CJ7, D3 and R1; data not shown). The remaining population was a mixture of cells with a variety of morphologies. The small elongated cells had similar morphological features to native neuroepithelial precursor cells (Collazo et al., 1992; Vicario-Abejon et al., 1995) and were selectively stained with an antibody against the intermediate filament protein nestin (Fig. 1B) (Frederiksen and McKay, 1988; Lendahl et al., 1990) and an antibody against brain fatty acid binding protein (data not shown) (Kurtz et al., 1994), both of which are expressed in neuroepithelium in vivo. These antibodies did not stain other cell types in the culture, nor did they stain ES cells before differentiation. Tightly-packed epithelial cells were nestin-negative (Fig. 1D) but reacted with a keratin 8 antibody (Fig. 1C). The nestin-positive, small elongated cells (Fig. 1D) were keratin 8-negative (Fig. 1C). These results are consistent with the fact that keratin 8 is expressed in the immature skin but not in the neural tube (Kemler et al., 1981). To see whether each supplement in ITSFn medium is necessary for the selection of neuronal precursor-like cells, attached EBs were maintained for 6 days with different combinations of supplements, replated as dissociated cells, and stained with an anti-nestin antibody. Both transferrin and fibronectin had general effects on cell survival, whereas the effect of insulin was more specific to the survival of nestin-positive cells (Table 1). The proportion of nestin-positive cells seen in these cultures is similar to that seen in vivo (Frederiksen and McKay, 1988). This data is consistent with the hypothesis that the major cell type generated in ITSFn medium has the properties of neuronal precursor cells.

2.2. Proliferation of neuronal precursor cells in the presence of bFGF

Previous studies show that bFGF is a strong mitogen for neuroepithelial precursor cells (Gensburger et al., 1987; Cattaneo and McKay, 1990; Kilpatrick and Bartlet, 1993; Ray et al., 1993; Ghosh and Greenberg, 1995; Vicario-Abejon et al., 1995). To investigate the response of EB-derived cells to bFGF, J1 ES cells kept in ITSFn medium for 6–7 days were dissociated and plated in several different DMEM/F12-based media. Three days later, cell density was measured. As shown in Table 2, a combination of DMEM/F12 medium supplemented with modified N3 (mN3 medium), bFGF and fibronectin had the highest proliferative effect. At the range of concentration from 5 to 50 ng/ml, bFGF showed the same effect on proliferation. At the concentration lower than 1 ng/ml, bFGF did not show clear proliferative effects. Since laminin showed a slightly higher stimulation of cell proliferation than fibronectin (Table 3), a combination of mN3 medium, bFGF and laminin (mN3FL medium) was used as a proliferation condition for neuronal precursor-like cells.

In mN3FL medium, the predominant proliferating cells resembled ITSFn medium-induced nestin-positive cells (Fig. 2A). When grown in mN3FL medium, other ES cell lines (D3, CJ7 and R1) took on the same morphology as the J1 cell line (Fig. 2B) and proliferation was also strictly dependent on bFGF. The cell proliferation was quantitated by counting the cell density 1, 4 and 7 days after plating. Cell counting showed a six-fold increase in cell number after 7 days in culture (Fig. 3A). We also stained the preparation with antibodies specific to neuronal precursors (nestin) (Fig. 2C), post-mitotic neurons (microtubule-associated protein 2; MAP2) (DeCamilli et al., 1984) (Fig. 2C), astrocytes (glial fibrillary acidic protein; GFAP), and oligodendrocyte-lineage cells (O4 (Sommer and Schachner, 1981), Gal-C). Nestin-positive cells were >80% of the total cell population at each time point. The other major cell type was MAP2-positive cells (10–15%) (Fig. 3A). We observed a small number of GFAP-positive cells (<2%), but no O4- or Gal-C-positive cells in this preparation.

To confirm the proliferation of nestin-positive cells, differentiated J1 and R1 cells were pulse-labeled with 5′-bromo-2′-deoxyuridine (BrdU) for 8 h and double-stained with both anti-BrdU antibody and eith r anti-nestin (Fig. 2D) or anti-MAP2 antibody. Cell counting revealed that

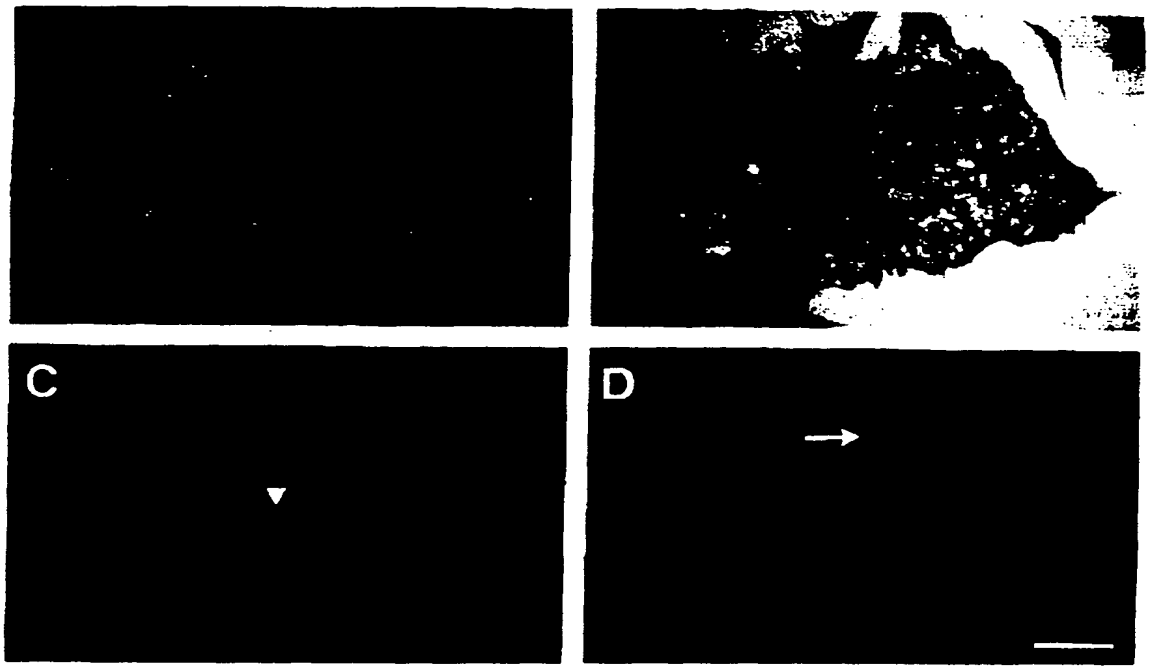


Fig. 1. Morphology and immunoreactivity of spreading EBs derived from J1 ES cells. EBs which attached to the culture substrate were cultured in ITSFn medium for 7 days, fixed and stained with anti-nestin (B,D) or anti-keratin 8 (C). Each pair of images ((A) and (B), (C) and (D)) is from the same field. Nestin-positive cells have elongated morphology (A). When cells are relatively sparse, rosette-like organization of 5–50 cells were frequently observed (arrow in (D)). These cells were immunoreactive with an anti-nestin antibody (D). Tightly-packed, epithelium-like cells remaining in the middle of nestin-positive cells were immunoreactive with keratin 8 (arrowhead in (C)). Scale bar, 100 μm.

Fig. 2. Morphology of nestin-positive, proliferating cells in mN3FL medium. (A,B) Phase contrast images of J1 (A) and CJ7 (B) cells proliferating in mN3FL medium. (C) J1 ES cells double stained with nestin (red color) and MAP2 (dark purple color) antibodies. Most cells are nestin-positive and MAP2-positive cells are few. Arrow indicates a MAP2-positive cell which has no extending processes. (D) Cells double stained with nestin (red color) and BrdU (dark purple color) antibodies. Cells were incubated with BrdU for 8 h before fixation. It is possible to identify cells either double stained with nestin and BrdU (arrow) or stained only with nestin (arrowhead). Scale bar, 100 μm for (A,B), 67 μm for (C,D).

Table 1
Effects of each component of ITSFn medium on the density of nestin-positive cells

Medium components	Cell density (cells × $10^3/cm^2$) (mean ± SEM)	Nestin-positive cells (%)
Insulin and selenium	20.6 ± 2.9	81.1
Transferrin and selenium	14.2 ± 2.5	19.7
Insulin, transferrin and selenium	23.2 ± 3.8	70.3
Insulin, transferrin, selenium and fibronectin	27.6 ± 3.3	84.1

J1 ES cells were attached to the culture substrate and maintained in several different conditions for 6 days, dissociated and replated in mN3FL medium for 1 day, and fixed for staining with anti-nestin antibody.

Table 3
Effects of cell adhesion molecules on the increase of cell density in mN3FL medium

Condition	Cell density (cells × $10^3/cm^2$) (mean ± SEM)	Increase of cell density from day 0 (%)
Day 0	21 ± 4.7	NA
Day 5 in mN3 medium plus bFGF and laminin	97.5 ± 5.6	+364
Day 5 in mN3 medium plus bFGF and fibronectin	89 ± 6.2	+324

J1 ES cells in ITSFn medium for 6–8 days were dissociated, plated in modified N3 medium plus 5 ng/ml bFGF, plus either 1 μg/ml of laminin or 1 μg/ml of fibronectin. After 5 days in culture, cell density was measured.

90% of BrdU-positive cells were nestin-positive and <1% of BrdU-positive cells were MAP2-positive (Fig. 3C,D). These immunocytochemical and BrdU labeling experiments suggest that nestin-positive cells derived from ES cells in vitro divide to generate nestin-positive progeny and this process is bFGF-dependent. This result is consistent with data obtained from the culture of primary cells from the CNS (Gensburger et al., 1987; Cattaneo and McKay, 1990; Ray et al., 1993; Ghosh and Greenberg, 1995; Vicario-Abejon et al., 1995) but distinct from results in the peripheral nervous system (PNS) (Zackenfels et al., 1995).

2.3. Differentiation of neuronal precursor cells toward neuronal and glial lineages

The direct demonstration of the state of neuronal precursors is to show that these cells actually become postmitotic neurons. We have previously shown that proliferating primary neuroepithelial cells differentiated into neurons when the mitogen was withdrawn (Cattaneo and McKay, 1990). To enhance neuronal differentiation, differentiated J1 cells maintained in mN3FL medium for 7 days were kept in mN3 medium plus laminin but without bFGF another 4 days. Cell counting of these preparations at 7 and 11 days showed a four-fold increase of MAP2-positive cells and three-fold decrease of nestin-positive cells, resulting in a cell population containing >60% of MAP2-positive cells (Fig. 3B). The CJ7 ES cell line showed a similar proportion of nestin- versus MAP2-positive cells after withdrawal of bFGF (Fig. 3E). MAP2-positive cells after withdrawal of bFGF had a morphology of immature neurons. The density of MAP2-positive cells after differentiation was three-fold higher than the initial density of the total cell population (Fig. 3B). This indicates that at least two-thirds of the MAP2-positive cells were newly generated during the bFGF-induced proliferative phase. Since we have shown that 90% of the dividing cells are nestin-positive (Fig. 3C, D), these cells are likely to be the precursors of the MAP2-positive, neuron-like cells induced by withdrawal of bFGF.

Table 2
Effects of each component of mN3FL medium on the increase of cell density

Condition	Cell density (cells × $10^3/cm^2$) (mean ± SEM)	Increase of cell density from day 0 (%)
Day 0	26.5 ± 3.8	NA
Day 3 in ITSFn	12.5 ± 2.2	−52.8
Day 3 in mN3 plus fibronectin	17.5 ± 2.1	−34.0
Day 3 in mN3 plus bFGF	43.5 ± 4.4	+64.2
Day 3 in mN3 plus bFGF and fibronectin	60 ± 5.3	+126

J1 ES cells in ITSFn medium for 6 days were dissociated, plated in four different media, maintained for 3 days and then counted for total cell density. bFGF has a major effect on cell proliferation since only mN3 plus bFGF and mN3 plus bFGF and fibronectin media promoted net increase of cell number.

Differentiation toward glial cell lineages was analyzed by staining cells with anti-GFAP or anti-O4 antibodies. Fig. 4A,B shows GFAP-positive cells with the characteristic morphology of cultured astrocytes. Cell counting showed that GFAP-positive cells were 10–15% of the total cell population. We could observe no O4-positive, oligodendrocyte-like cells by simple withdrawal of bFGF.

Previous reports on O2A progenitor cells showed that thyroid hormone stimulates the differentiation of oligodendrocytes (Barres et al., 1994). The effect of thyroid hormone was tested by switching the medium to mN3 medium with tri-iodothyronine (T3) but without bFGF for 6 days. We could identify a small number of O4-positive cells (1–2% of the total cell population), which had typical oligodendrocyte morphologies (Fig. 4C,D). T3 did not influence the number of MAP2-positive or GFAP-

positive cells. These results suggest that the ES cell-derived nestin-positive cell population contains precursors which give rise to neurons, astrocytes and oligodendrocytes.

2.4. Further maturation of neuronal cells derived from ES cells

The neuronal cells induced to differentiate by the withdrawal of bFGF could be maintained without significant cell death in neurobasal medium plus B27 supplement and 5% fetal calf serum for more than 2 weeks. This long-term culture was successfully applied to J1, CJ7 and D3 cell lines. In contrast, long-term culture was difficult in N3-based serum-free medium.

An immunocytochemical analysis of these neuronal cells was performed. Double-labeling of MAP2 and neurofilament-M (NF-M) indicated that two classes of neurite were present (Fig. 5A,B). The anti-MAP2 antibody stained short thick processes and cell bodies while anti-NF-M revealed thin, long processes. These results suggest that ES cell-derived neurons had MAP2-positive dendrites (Caceres et al., 1984) and NF-M-positive axons (Shaw et al., 1985). Anti-synapsin I staining revealed punctate structures closely associated with the plasmalemma of dendrites (Fig. 5C). The staining pattern suggests segregation of synaptic vesicles to distinct sites along the axons (Fletcher et al., 1991). To investigate the transmitter phenotypes, cells were stained with several antibodies against neurotransmitters. There were a large number of glutamate-positive cells (Fig. 5D), mixed with completely negative cells. γ-Aminobutyric acid (GABA)-positive cells were also common (Fig. 5E,F) and GABA staining was also confined to a subset of neurons. It was possible to identify thin processes which were GABA-positive but MAP2-negative. This further suggests the differentiation of the dendritic and axonal structures, since the axons of GABAergic neurons should be GABA-positive and MAP2-negative. We could not detect choline acetyltransferase (ChAT) immunoreactivity in our cell preparation. Since this antibody could detect cholinergic neurons in vivo and in culture, the level of ChAT in ES cell-derived neurons was much lower than that in authentic cholinergic neurons.

Neuronal gene expression was further analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using a panel of neuron-specific primers (Fig. 6A). The

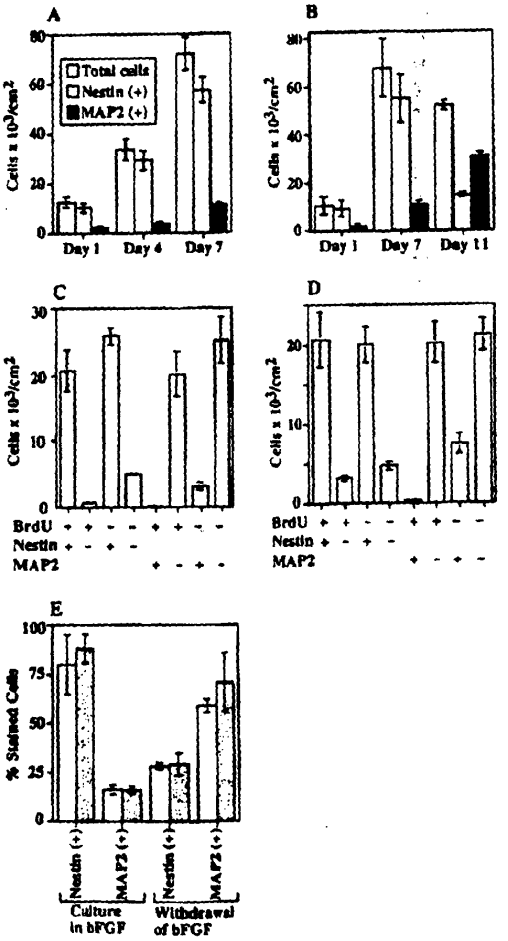


Fig. 3. Proliferation and differentiation of nestin-positive cells derived from J1, CJ7 and R1 ES cells. (A) Time course of proliferation in mN3FL medium. Differentiated J1 ES cells were dissociated and replated. At 1, 4 and 7 days later, cells were fixed, stained with anti-nestin and anti-MAP2 antibody, and the density of total cells, nestin-positive cells, and MAP2-positive cells was measured. After 7 days in culture, the total cell density increased six-fold, and both the nestin-positive cell density and the MAP2-positive cell density increased five-fold. (B) Induction of MAP2-positive cells by removing bFGF from the medium. Differentiated J1 ES cells were incubated in mN3FL medium for 7 days until the culture became confluent, and bFGF was removed. The cell density for the total cell population, the nestin-positive cells, and the MAP2-positive cells the day after plating, 7 days after plating, and 4 days after differentiation (J1 days after plating) is shown. The increase of MAP2-positive cells is evident after the washout of bFGF. The number of nestin-positive cells is down-regulated. (C,D) Incorporation of BrdU into nestin-positive/negative or MAP2-positive/negative populations. Differentiated ES cells (J1 ES cells in (C) and R1 ES cells in (D)) in mN3FL medium were incubated with BrdU for 8 h, fixed immediately after washing out BrdU and double-stained with anti-BrdU antibody and either anti-nestin or anti-MAP2 antibody. Cells positive for both nestin and BrdU were approximately 40% of the total cell population. In contrast, cells double-positive for MAP2 and BrdU were <1% of total cells. (E) Proportion of nestin-positive and MAP2-positive cells before and after differentiation. Differentiated J1 (open bar) and CJ7 (shaded bar) ES cells were allowed to proliferate for 5–7 days and the bFGF was withdrawn. The proportions of nestin and MAP2-positive cells were determined before the withdrawal of bFGF and 4–5 days after the withdrawal. The nestin-positive cells decreased threefold and the MAP2-positive cells increased three- to four-fold through the differentiation.

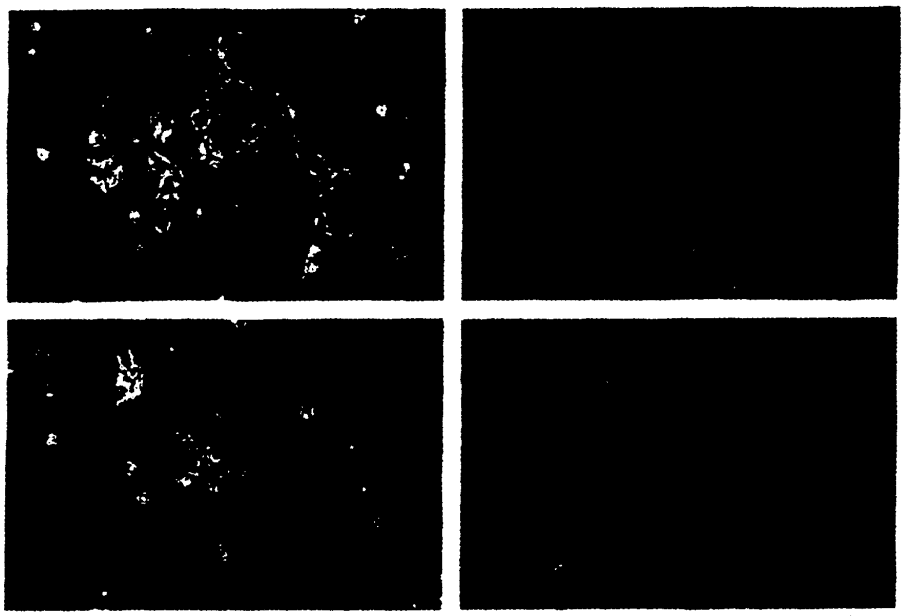


Fig. 4. Differentiation of glial cells from J1 ES cells. (A,B) Phase (A) and bright field (B) images of GFAP-positive cells derived from ES cells. Differentiation was induced by withdrawing bFGF from the culture of proliferating nestin-positive cell population. (C,D) Phase (C) and bright field (D) images of O4-positive cells derived from ES cells. Differentiation was induced by withdrawing bFGF and adding T3 for 6 days. A small proportion of ES cells was induced to be O4-positive cells which resembled the native oligodendrocytes in terms of their shape in culture (arrows). Scale bar, 50 μm.

preparation contained cells expressing glutamate decarboxylase (GAD_{65}), calbindin D_{28}, NMDA receptors 1, 2A, 2B, 2D, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, and $GABA_A$ receptor $\alpha 3$. In every case, much higher amounts of transcripts were detected in total RNA from differentiated cells.

To investigate whether these neuronal cells correspond to cells at any specific CNS regions, expression of three position-specific markers along the anterior-posterior axis was analyzed (Fig. 6B). Otx-1 is mainly expressed in forebrain and midbrain (Simeone et al., 1992), En-1 in midbrain-hindbrain boundary (Joyner and Martin, 1987), and Hoxa-7 in the posterior spinal cord (Mahon et al., 1988). Undifferentiated ES cells expressed Hoxa-7 but no Otx-1 and En-1 expression was detected. The expression of the posterior marker Hoxa-7 was down-regulated in nestin-positive cells proliferating in the presence of bFGF for more than 10 days. In contrast, Otx-1 and En-1 were up-regulated in these proliferating cells. After differentiation by switching to Neurobasal medium plus B27 and serum, Hoxa-7 expression was up-regulated again and Otx-1 and En-1 expression was maintained. The presence of these transcriptional factors suggests that this preparation contains neurons from different regions of the developing brain. These results collectively suggest that this preparation generates neurons characteristic of different CNS regions.

2.5. Functional characterization of neuronal cells derived from ES cells

To characterize electrophysiological properties, neuronal cells were maintained in neurobasal medium plus B27 and 5% FCS for >12 days and activity of 15 cells was recorded from three plates. The resting membrane potentials were about –60 mV. All the cells exhibited inward action current upon depolarization by 20 mV from resting potential. These inward currents were followed by a fast inactivating outward current (I_A) and a sustained outward current (Fig. 7A–C). These currents were absent in Cs^+-filled cells, indicating that they were likely to be mediated by outward K^+-rectifying channels.

Most of the cells expressed spontaneous synaptic currents of varying durations and magnitudes. Application of glutamate onto adjacent, putative neurons could trigger the generation of these synaptic currents in the recorded neuron with a short delay (Fig. 7D). The recorded synaptic currents were of two types, fast excitatory postsynaptic currents, reversing at about 0 mV, and slow-decaying inhibitory synaptic currents, reversing at about -50 mV, when recorded in acetate-containing pipettes (Fig. 7D). The recorded cells themselves responded to topical application of glutamate with a marked inward current recorded at resting potential. The spontaneous and evoked synaptic responses, as well as the responses of the cells to

glutamate, indicate that the recorded cells in culture maintain an array of properties akin to those of prenatal, cultured CNS neurons (Segal, 1983; Segal and Barker, 1984).

NMDA stimulation induces the phosphorylation of the cyclic adenosine monophosphate response element binding protein (CREB protein) and transcription of the c-fos gene (Bading et al., 1993; Ginty et al., 1993). These two inductions were analyzed to determine whether functional NMDA receptors were expressed. Unstimulated cells did not show any phospho-CREB staining (Fig. 8A,B). In contrast, 10 min after stimulation with either glutamate (Fig. 8C,D) or NMDA (Fig. 8E,F), cells showed intense nuclear immunoreactivity. Phospho-CREB staining was confined to the neurons. Large nuclei of glia-like cells showed no phospho-CREB staining (arrowheads in Fig. 8C–F). RT-PCR of cells treated with glutamate or NMDA revealed c-fos induction, whereas untreated cells showed no c-fos induction (data not shown). These results strongly suggest that some of the neurons in this preparation have functional NMDA receptors.

The presence of synaptic connections was confirmed

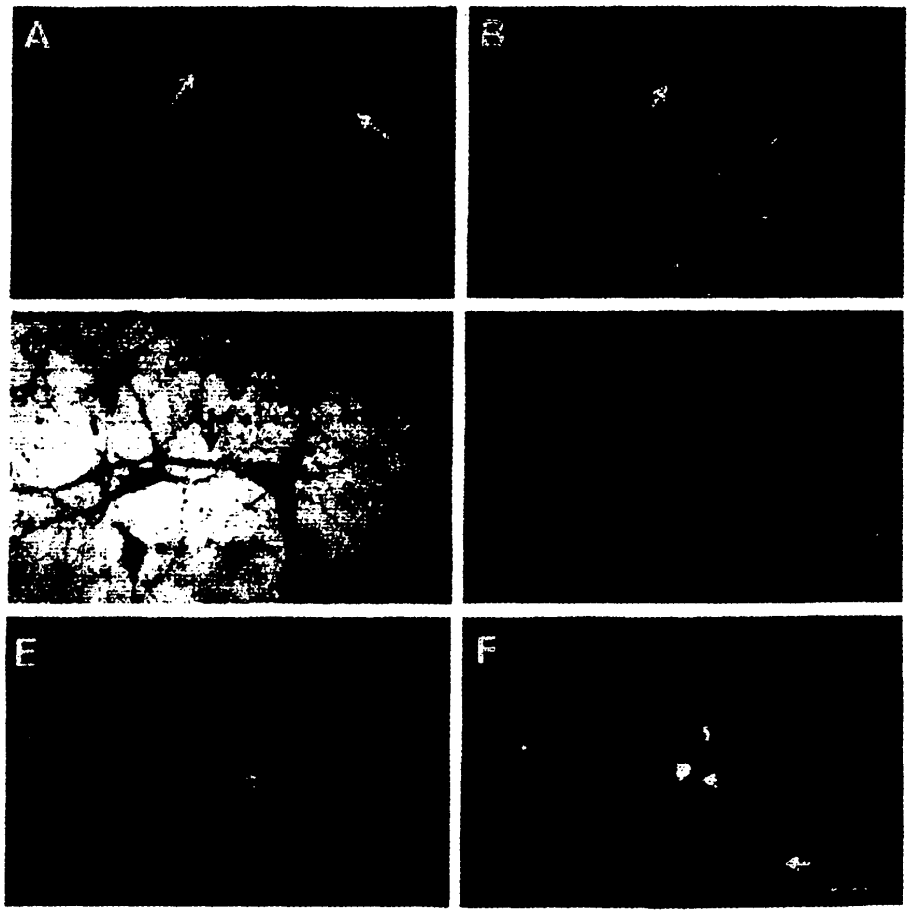


Fig. 5. Immunocytochemical characterization of neuron-like cells differentiated from J1 ES cells. Cells were incubated in ITSFn medium for 6–8 days, dissociated, plated in mN3FL medium, passaged twice, and switched to neurobasal medium plus B27 and 5% FCS. Cells were maintained for 10–20 days and processed for immunocytochemistry. (A,B) Neuron-like cells double-stained with anti-MAP2 antibody (A) and anti-NF-M antibody (B). In this double label experiment, short processes and cell bodies are immunoreactive only with MAP2 (arrows in (A)) and long, straight processes are only immunoreactive with NF-M (arrow in (B)). (C) Anti-synapsin I reveals punctate presynaptic structure along the dendrites (arrow). (D) A phase contrast micrograph of cells stained with anti-glutamate antibody. Immunoreactive cell bodies (arrow) and immunonegative cell bodies (arrowhead) can be identified. (E,F) Double-label immunocytochemistry of neuron-like cells stained with anti-MAP2 antibody (E) and anti-GABA antibody (F). Some cells are immunoreactive with both MAP2 and GABA antibodies (arrowheads) and other cells are immunoreactive only with MAP2 (large arrows). Thin, branching processes which are MAP2-negative and GABA-positive (small arrows) are frequently observed, possibly GABAergic axon-like neurites. Scale bar, 50 μm for (A,B,D–F), 31 μm for (C).

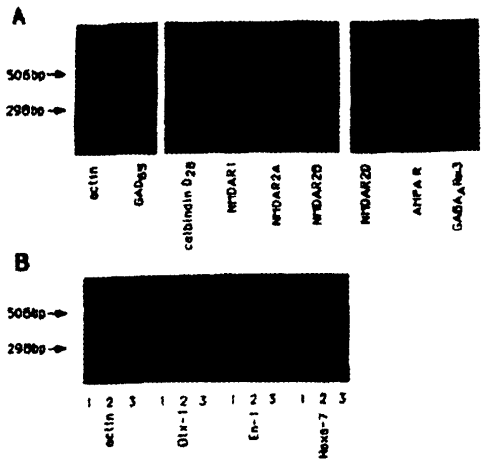


Fig. 6. RT-PCR analysis of the pattern of gene expression in undifferentiated and differentiated J1 ES cells. (A) Expression of neuron-specific genes. Total RNA was extracted from either undifferentiated ES cells or ES cells differentiated in neurobasal plus B27 and 5% FCS. Samples from undifferentiated (left lane) and differentiated (right lane) cells were loaded in adjacent lanes. (B) Expression of position-specific markers along the anterior-posterior axis. Total RNA from undifferentiated ES cells (1), nestin-positive cells which had been proliferated for 12 days in the presence of bFGF (2), and cells differentiated in neurobasal plus B27 and 5% FCS for 10 days (3) was reverse transcribed and amplified.

by electron microscopy. Typical pre-synaptic structures containing numerous synaptic vesicles were found to be associated with dendrites (Fig. 9). Thickening of the membrane, which is characteristic for the active zone, was also observed. We conclude that neuronal precursor cells derived from ES cells can be differentiated into post-mitotic neurons which form functional synaptic connections.

3. Discussion

The data presented in this study demonstrate that ES cells can generate proliferating neuronal precursor cells which can be expanded and differentiate efficiently to synaptically connected neurons and glia. This study shows that the lineage restriction observed in the developing embryo can be replicated in the in vitro culture system in a precisely determined manner. We could effectively separate the state of pluripotent cells, the state of proliferating neuronal precursors and the final differentiation toward neurons and glia by using a combination of serum-free culture conditions and growth factors. The fact that ES cell-derived neuronal precursor cells share common properties with authentic precursors shows that the molecular mechanisms involved in the development of CNS cells in vivo also function in our culture system. The in vitro system described here is, to our knowledge, the first demonstration that the proliferating neuroepithelial precursor cells can be generated with high yield from pluripotent stem cell lines.

3.1. Generation of bFGF-responsive, CNS precursor cells from ES cells

Several lines of evidence suggest that the ES cell-derived neuronal precursor cells correspond to CNS neuroepithelial precursor cells. Previous studies on primary cultures of CNS neuroepithelial cells have shown that bFGF can stimulate proliferation of this cell type (Ghosh and Greenberg, 1995; Ray et al., 1993; Vicario-Abejon et al., 1995). In contrast, bFGF alone does not support the proliferation of PNS precursor cells in vitro (Stemple and Anderson, 1992). The similarity of the effect of bFGF on both ES cell-derived neuronal precursor cells and CNS primary cells suggests common underlying mechanisms of bFGF action. The characterization of ES cell-derived neuronal cells also supports the CNS-like characters of these cells. Namely, these neurons are immunoreactive with anti-glutamate and anti-GABA antibodies, and express mRNA of glutamate receptors and GABA receptors.

The mechanism of region-specific differentiation of neuroepithelial cells is currently one of the central questions in developmental neurobiology. Presence of both anterior and posterior markers in the nestin-positive cell population indicates that ES cells can generate neurons from both anterior and posterior brain regions. These induced phenotypes are likely to be altered by switching culture medium, since Hoxa-7 was up-regulated after differentiation in serum-containing medium. It is possible that the induction of Hoxa-7 is due to a retinoic acid-derivative which is included in B27 supplement, since it is known that the application of retinoic acid to embryonal carcinoma cells can induce Hox gene corresponding to a more posterior region (Simeone et al., 1990).

Members of the FGF family are known for their diverse roles in early embryonic development. As discussed previously, one of the major effects is on the proliferation of a variety of cell types, including neuronal precursor cells. Recently, bFGF has been shown to play a role on the induction of posterior neural structures in *Xenopus* embryos (Doniach, 1995). In contrast, we observed down-regulation of posterior neural marker Hoxa-7 in cells proliferated in the presence of bFGF. Furthermore, the anterior marker Otx-1 was still expressed. We also could not detect ChAT-positive neurons, suggesting a lack of motor neuron differentiation in our culture system. These results suggest that bFGF does not drive mammalian neuroepithelial cells to a posterior fate.

Cultures of *Xenopus* animal caps show that another major role of FGF receptor signaling is in mesoderm in-

duction (Kimelman and Kirschner, 1987). Mice lacking FGF receptor 1 showed altered patterning of mesoderm structures during gastrulation (Deng et al., 1994; Yamaguchi et al., 1994). The analysis of the role of FGF in the neuroectoderm was compromised by the disruption of axial mesoderm which is known to produce neural inducers. By the in vitro differentiation of ES cells carrying mutant FGF ligand and/or receptor genes, it will be possible to analyze the effects of deletion of the FGF signaling system on the neuroectoderm induction and subsequent development of CNS structures.

3.2. *Nestin-positive cells derived from ES cells can differentiate into functional post-mitotic neurons*

The neuronal cells generated from ES cells in our culture system fulfill the criteria of functional post-mitotic neurons. First, they develop specialized structures such as dendrites and the axon and compartmentalize cytoskeletal proteins to specific regions. Second, the cells express a variety of neurotransmitters, transmitter receptors and ion channels. Third, functional synaptic contacts are formed between these cells. Fourth, these neurons can activate the signaling pathways downstream from the glutamate receptors. These findings lead us to the conclusion that functional neurons are generated from ES cells in vitro.

It should be emphasized that our culture system can induce highly enriched post-mitotic neurons from proliferating precursors in a growth factor-dependent manner. Previous studies have shown that ES cells can differentiate into post-mitotic neurons by the treatment with retinoic acid in a serum-containing medium (Bain et al., 1995; Finley et al., 1996; Fraichard et al., 1995). Although these studies have presented evidence that a subpopulation of cells formed functional synaptic connections, the proportion of neuroectodermal lineage cells is relatively small and the differentiation toward neurons and glia was not controlled by growth factors. In the system described here, up to 95% of the cells are stained with the neuroepithelial precursor cell marker nestin in

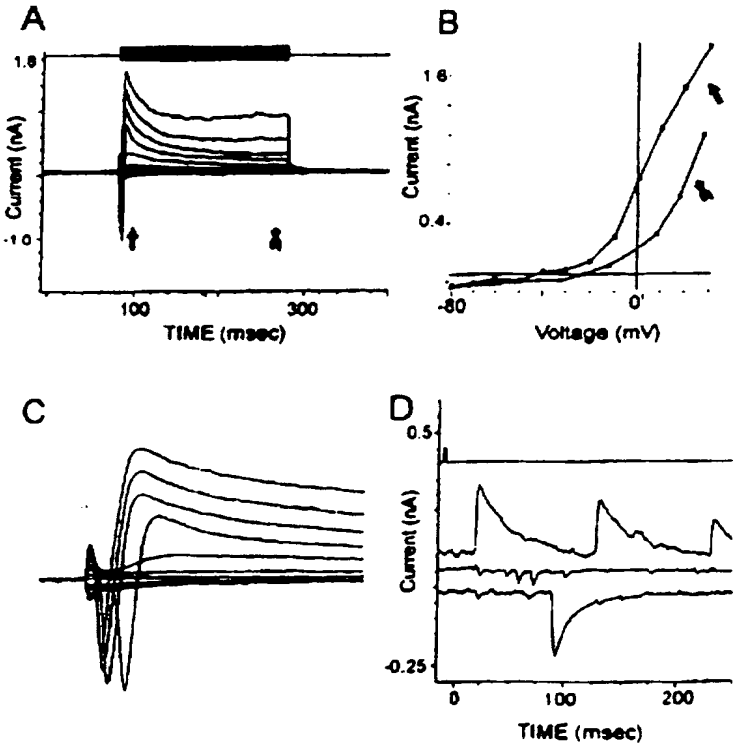


Fig. 7. Intrinsic and synaptic properties of differentiated J1 ES cells. (A) Current responses to a series of voltage commands evoked from a holding potential of −60 mV in a patch-clamped neuron. From resting potential, no current responses to hyperpolarizing voltage commands were seen. In response to depolarizing voltage commands, an inward current followed by fast and slow outward currents was seen. (B) Current-voltage curves from the cell shown in (A), at two different time points indicated by the arrows. The fast current has a lower activation threshold and higher conductance. (C) Expansion of the traces in (A), to illustrate the action currents generated in the cell upon depolarizing voltage commands. (D) Inward and large outward synaptic currents evoked in the cell upon application of a pulse of glutamate onto an adjacent cell (top line). The recorded cell was held at different potentials, −90, −50, and 0 mV, and the evoked synaptic currents showed a slow inactivation, especially pronounced at the positive holding current, but note the train of fast, small inward currents, seen at membrane potential where the large (presumably $GABA_A$-mediated) synaptic currents were at the reversal potential (−50 mV).

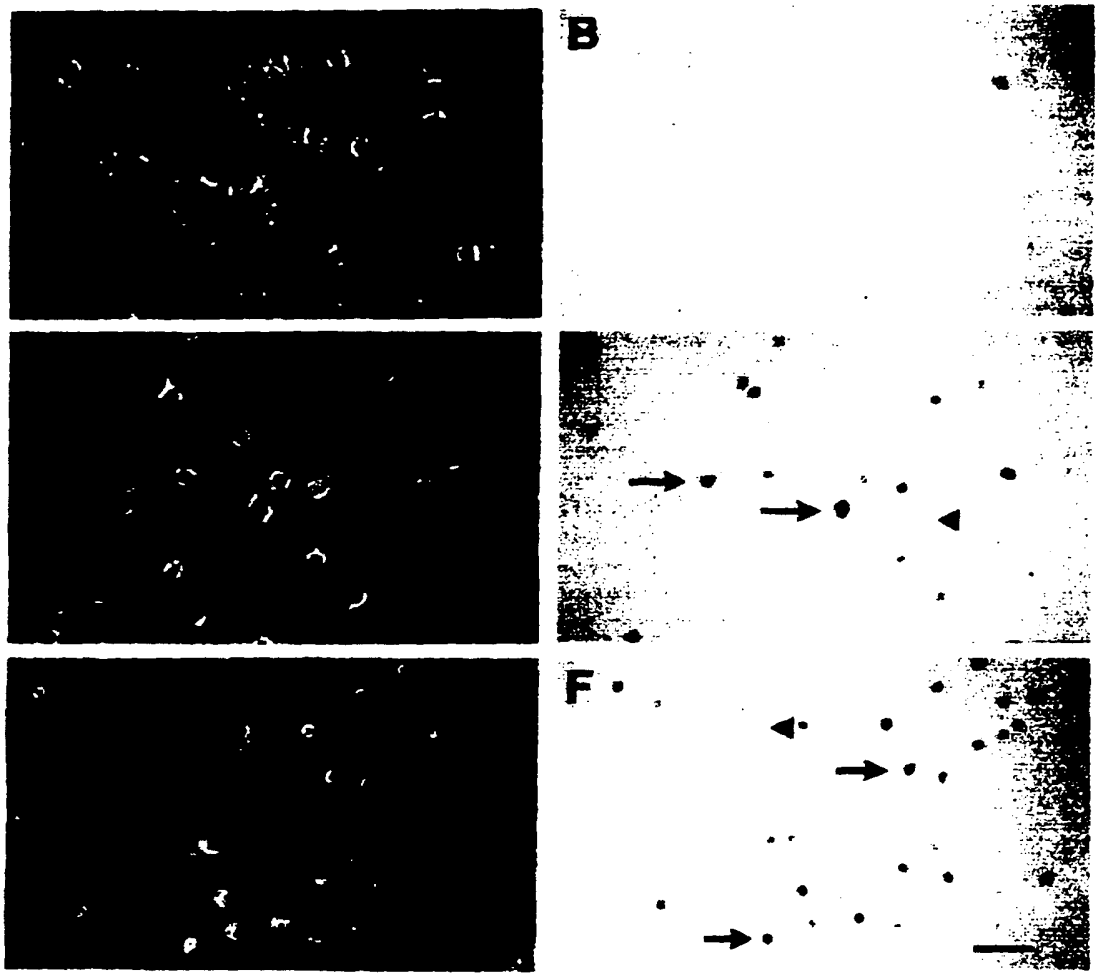


Fig. 8. Induction of phosphorylated CREB immunoreactivity by the activation of glutamate receptors. Phase images (A,C,E) and bright field images (B,D,F) of cells differentiated in neurobasal medium plus B27 and 5% FCS for 2 weeks are shown. Cells were stimulated with 10 μM glutamate (C,D) or 10 μM NMDA (E,F) for 10 min. Unstimulated cells showed no nuclear staining with anti-phosphorylated CREB antibody (A,B). Samples stimulated with either glutamate or NMDA contained many neuron-like cells with intense nuclear immunoreactivity (arrows in (C–F)). In contrast, glia-like flat cells did not show any nuclear staining (arrowheads in (C–F)). Bar, 50 μm.

the proliferation phase and >60% of the cells are induced to be MAP2-positive neuronal cells after differentiation. The highly enriched population of neuronal lineage cells in serum-free culture system permits the biochemical analysis of signaling mechanism through growth factor receptors and neurotransmitter receptors.

The data presented in this paper show that multiple steps of the in vivo development of CNS can be effectively analyzed in culture. In combination with the methods to introduce mutations to specific genes (Mortensen et al., 1992), this approach will be useful for the functional analysis of a variety of mutations systematically created in specific genes or for screening a large number of gen -trap ES cell clones by functional crit ria.

4. Experimental procedures

4.1. Materials

The materials were purchased from the following sources: fibronectin, laminin, neurobasal medium, B27 supplement, and superscript II RNase H⁻ reverse transcriptase from Gibco/BRL (Grand Island, NY); bFGF from R&D Systems (Minneapolis, MN); insulin, transferrin, s lenium chloride, polyornithine, progesterone, putrescine, T3, cytosine arabinoside, anti-MAP2 antibody, anti-NF-M antibody, anti-GABA antibody, and anti-glutamat antibody from Sigma (St. Louis, MO); Taq polymerase from Boehringer-Mannheim (Mannheim,

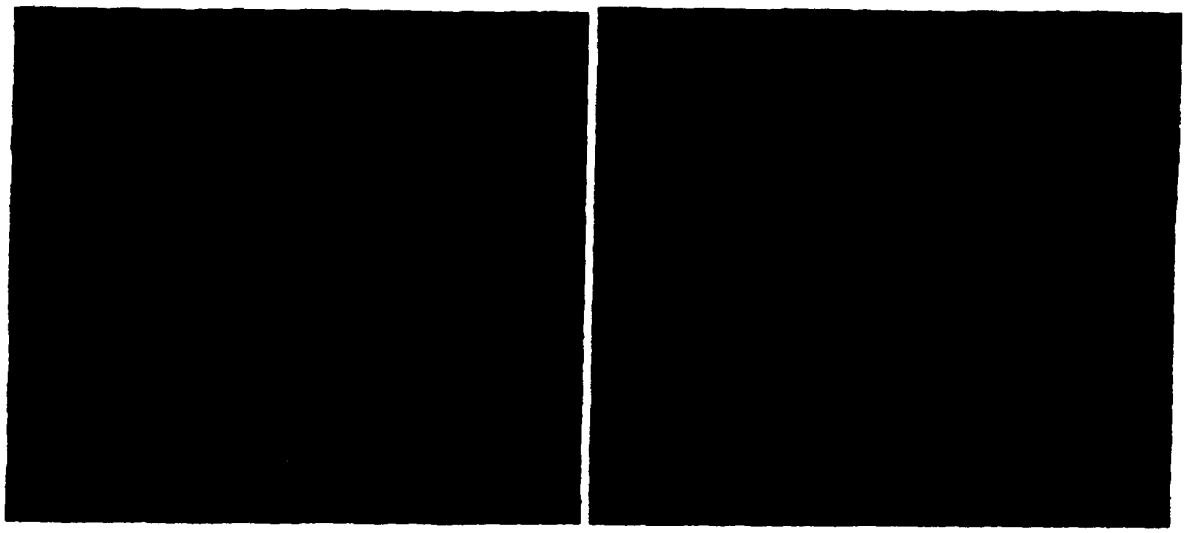


Fig. 9. Electron microscopy of synaptic structures formed between differentiated neuron-like cells derived from J1 ES cells. Presynaptic structures filled with synaptic vesicles (arrows) are closely apposed to postsynaptic membranes which frequently show postsynaptic density-like membrane thickening (double arrows). Scale bar, 100 nm.

Germany); Anti-GFAP antibody from ICN Biomedicals (Costa Mesa, CA); anti-keratin 8 antibody from American Type Culture Collection (Rockvillle, MD); Vectastain ABC kit from Vector laboratories (Burlingame, CA); double staining kit and aminoethyl carbazole from Zymed Laboratories Inc. (Carlton Court, CA); anti-phosphorylated CREB antibody from Upstate Biotechnology Inc. (Lake Placid, NY); BrdU staining kit from Amersham (Arlington Heights, IL); all fluorescence secondary antibodies from Cappel (Durham, NC).

4.2. Cell culture

4.2.1. Maintenance of ES cell lines and embryoid body (EB) formation

Maintenance of embryonic stem cell lines (J1, CJ7, D3, and R1) (Doetschman et al., 1985; Lee et al., 1992; Nagy et al., 1993; Swiatek and Gridley, 1993) and EB formation were as previously described (Robertson, 1987).

4.2.2. Selection of nestin-positive cells

Embryoid bodies kept in suspension culture for 4 days were plated onto a tissue culture surface. A 24 h incubation in DMEM-10% FCS medium allowed EBs to spread on this substrate. The next day the medium was switched to DMEM/F12 (1:1) supplemented with insulin (5 μg/ml), transferrin (50 μg/ml), selenium chloride (30 nM), and fibronectin (5 μg/ml) (ITSFn medium). The culture medium was replenished every 2 days. A maximal number of nestin-positive cells appeared approximately 6–8 days after replacement with ITSFn medium.

4.2.3. Expansion of nestin-positive cells by bFGF

Cells maintained in ITSFn medium were dissociated by 0.05% trypsin and 0.04% EDTA in PBS, neutralized with DMEM/F12 (1:1) plus 10% FCS, collected by centrifugation, and replated at a cell density of 0.5–2 × 10^5/ cm^2 on dishes precoated with polyornithine (15 μg/ml) and laminin (1 μg/ml). Culture medium was DMEM/F12 supplemented with insulin (25 μg/ml), transferrin (50 μg/ ml), progesterone (20 nM), putrescine (100 μM), selenium chloride (30 nM), bFGF (5 ng/ml), and laminin (1 μg/ml) (mN3FL medium containing bFGF and laminin, mN3 medium without bFGF and laminin). The medium was changed every 2 days. For passage, cells were dissociated by 0.05% trypsin and 0.04% EDTA in PBS, collected by centrifugation, and replated.

4.2.4. Differentiation of nestin positive cells expanded by bFGF

Differentiation of nestin-positive cells was induced by 2 different methods. (a) To investigate early neuronal marker induction, proliferating nestin-positive cells in mN3FL medium were switched to mN3 medium with laminin. (b) T see long-term differentiation, cells proliferating in mN3FL medium were dissociated and plated onto either a polyornithine/laminin-coated surface or a primary glial cell monolayer prepared from neonatal mouse forebrain (Kimelberg et al., 1978). The clumps

were allowed to spread for 3–4 days in mN3FL medium, and then switched to neurobasal medium plus B27 supplement and 5% FCS. To prevent glial cell proliferation, 10 μM of cytosine arabinoside was added 2–3 days after replacement to serum-containing medium. One-fourth of the medium was changed every 5 days.

4.3. Immunocytochemistry

Cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 20–30 min, permeabilized with 0.2% Triton X-100 in PBS, and treated with 5% normal goat serum. The cells were incubated for 30 min–1 h with the primary antibodies against keratin 8 (supernatant of producing cells), nestin (1:1000; from Dr. M. Marvin, NIH), brain fatty acid binding protein (1:1000; from Dr. T. Müller, NIH), MAP2 (1:200), NF-M (1:100), Synapsin I (1:1000; from Dr. M.B. Kennedy, California Institute of Technology), GFAP (1:50), O4 (supernatant of producing cells; from Dr. R.H. Quarles, NIH), GalC (supernatant of producing cells; from Dr. M. Dubois-Dalcq, NIH), GABA (1:1000), and glutamate (1:500). After washing with PBS, cells were processed according to the method for the Vectastain ABC kit. For double immunofluorescence staining with MAP2 and NF-M, cells were fixed, permeabilized with Triton X-100, and treated with NGS in a similar manner. Then the cells were incubated with monoclonal anti-MAP2 antibody, followed by fluorescein-labeled anti-mouse IgG, and then fixed again with 2% paraformaldehyde for 30 min. After re-fixation, the cells were incubated with monoclonal anti NF-M antibody, followed by rhodamine-labeled anti-mouse IgG. The second fixation eliminates the cross-reaction of the rhodamine-conjugated anti-mouse IgG to the anti-MAP2 monoclonal antibody. For double-label immunocytochemistry with enzyme-linked secondary antibodies, we followed the instructions of the double staining kit (Zymed Laboratories, Inc.). Staining with anti-phosphorylated CREB antibody (1:1000) was as described by Ginty et al. (1993).

4.4. Proliferation assay

Cells were incubated with BrdU for 8 h at 37°C. After incubation, the cells were fixed immediately and processed according to the instruction of BrdU staining kit. After the color reaction, the cells were incubated with 0.8% hydrogen peroxide and 5% NGS in PBS for 30 min to inactivate HRP activity. After intense washing, they were processed for either anti-nestin or anti-MAP2 antibody staining to generate a reddish reaction product in the cytoplasm with aminoethyl carbazole.

4.5. Cell counting

Cell density was determined by counting the number of cells per field at 200× magnification. Eight fields were analyzed for each sample, and cell densities were calibrated and averaged. Each cell density presented in the figures and tables was the average and SEM of three independent experiments, except in Fig. 3A (five independent experiments).

4.6. RT-PCR

Total RNA was extracted from each cell preparation by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The total RNA was treated with RNase-free DNase, and cDNA synthesis was performed according to the instructions for superscript II RNase H$^-$ reverse transcriptase. PCR reaction was performed in PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin) containing 0.2 mM dNTP, 0.3 μM each of forward and reverse primers, and 0.25 U of Taq polymerase. Cycling parameters were denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 60 s. Cycling times were determined for each primer set to be within the exponential phase of amplification. Primer sequences and the length of amplified products are as follows (forward and reverse primers in this order): actin (ATGGATGACGATATCG CTG, ATGAGGTAGTCTGTCAGGT, 569 bp); NMDA R1 (ACCCTGTCCTCTGCCATGTGGTTTTC, ACATTC TTGATACCGAACCCATGTC, 591 bp); NMDA R2A (ACCCCAAGGACTGTAGTGAGGTTG, ATGTCATAG AGGTTGCCCATCCGCAG, 463 bp); NMDA R2B (ATGACTGTGACAACCCACCCTTT, ACTGACCGAA TCTCGCTTGAAGT, 494 bp); NMDA R2D (AACCAC TTCTTCTGCCAGGAGG, TGAAGGAGTCTGGGTTA TCCCA, 261 bp); calbindin D_{28} (ACCTGCAGTCATC TCTGATC, AGTTGCTGGCATCGAAAGAG, 276 bp); GAD_{65} (TCTTTTCTCCTGGTGGTGCC, TGCCTGAAG AAGTTCAATGC, 391 bp); $GABA_A\alpha 3$ (CCTTTGGCCT ATGAGATCTGGATGTG, TCGTACCACCATTTGTTT TTCA, 260 bp); AMPA receptor (CCTTTGGCCTATGA GATCTGGATGTG, TCGTACCACCATTTGTTTTTCA, 749 bp); Otx-1 (CACCCGGCTGTTAGCATGATGT, TA GACGAAGACGCAGAGCTAGA, 433 bp); En-1 (TGGT CAAGACTGACTCACAGCA, TCTCGTCTTTGTCCTG AACCGT, 389 bp); Hoxa-7 (TCTATCAGAGCCCCT TCGCGT, TGCCTGGCCCTTTACTCCTCTT, 520 bp); c-fos (GGCAAAGTAGAGCAGCTATC, AGGCCACAG ACATCTCCTCT, 270 bp).

Following primers amplify the cDNA fragments which contain splicing sites. Therefore, amplification of genomic DNA can be distinguished by the size of products: actin, NMDAR1, NMDAR2D, calbindin D_{28}, GAD_{65}, $GABA_A\alpha 3$, AMPA receptor.

For other primers, control amplification was done without adding reverse transcriptase to see any amplification of genomic DNA. No amplification of genomic DNA was observed in these control experiments.

4.7. Electrophysiology

Cells were recorded at room temperature with 3–6 MΩ patch pipettes containing (in mM) 130 K-acetate (or 120 CsCl + 10 KCl), 10 HEPES, 2 $MgCl_2$, 1 ATP, 0.1 EGTA, 10 NaCl. pH was adjusted to 7.2 with KOH, and osmolarity to 300 mosmol with sucrose. The recording saline contained (in mM) 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose. pH was adjusted to 7.4 with NaOH, and osmolarity to 320 mosmol with sucrose. Glutamate (1 mM, in the recording saline) was applied by pressure through a micropipette positioned near the recorded cell or near adjacent cells in the field of view, within 100 μm of the recorded cell. Current signals were amplified with an Axopatch amplifier, stored and analyzed on an IBM computer using pClamp-6 software.

4.8. Electron microscopy

Cells on plastic dishes were fixed with 2% paraformaldehyde and 1% glutaraldehyde in PBS for 1 h, washed with water, treated with 1% OsO_4, block-stained with uranyl acetate, dehydrated with ethanol and embedded in Araldite resin. Thin-sectioned samples were observed under JEOL 1200 EX electron microscope.

4.9. Stimulation of differentiated neuronal cultures

Cells differentiated in neurobasal medium plus B27 and 5% FCS were incubated with the same medium containing 10 μM of either glutamate or NMDA for 10 min. Cells were fixed immediately after stimulation for phospho-CREB staining. Cells were incubated for 50 min after stimulation and RNA was extracted for the analysis of c-fos induction.

Acknowledgments

We would like to thank to Dr. M.B. Kennedy (California Institute of Technology) for the gift of anti-synapsin I antibody; Dr. M. Marvin (NIH) for the anti-nestin antibody; Dr. T. Müller (NIH) for the anti-brain fatty acid binding protein antibody; Dr. R.H. Quarles (NIH) for the anti-O4 antibody; Dr. M. Dubois-Dalcq (NIH) for the anti-GalC antibody. We are particularly grateful to Dr. K.K. Johe and Dr. T.G. Hazel for sharing information on the culture conditions of neuronal precursor cells, Dr. K. Miyaguchi (NIH) for help in electron microscopic analysis, and David Liu for designing PCR primers for NMDA receptors. We also thank to Dr. J. Pickel for helping the initial setup of culturing CJ7 and R1 ES cell lines and Dr. P. Tsoulfas for his suggestions on the manuscript.

References

Bading, H., Ginty, D.D. and Greenberg, M.E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science 260, 181–186.

Bain, G., Kitchens, D., Yao, M., Huettner, J.E. and Gottlieb, D.I. (1995) Embryonic stem cells express neuronal properties in vitro. Dev. Biol. 168, 342–357.

Barres, B.A., Lazar, M.A. and Raff, M.C. (1994) A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development 120, 1097–1108.

Caceres, A., Banker, G., Steward, O., Binder, L. and Payne, M. (1984) MAP2 is localized to the dendrites of hippocampal neurons which develop in culture. Dev. Brain Res. 13, 314–318.

Cattaneo, E. and McKay, R.G. (1990) Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature 347, 762–765.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Collazo, D., Takahashi, H. and McKay, R.D.G. (1992) Cellular targets and trophic functions of neurotrophin-3 in the developing rat hippocampus. Neuron 9, 643–656.

DeCamilli, P., Miller, P.E., Navone, F., Theurkauf, W.E. and Vallee, R.B. (1984) Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence. Neuroscience 11, 817–846.

Deng, C.-X., Wynshaw-Boris, A., Shen, M.M., Daugherty, C., Ornitz, D.M. and Leder, P. (1994) Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev. 8, 3045–3057.

Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985) The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morphol. 87, 27–45.

Doniach, T. (1995) Basic FGF as an inducer of anteroposterior neural pattern. Cell 83, 1067–1070.

Finley, M.F.A., Kulkarni, N. and Huettner, J.E. (1996) Synapse formation and establishment of neuronal polarity by P19 embryonic carcinoma cells and embryonic stem cells. J. Neurosci. 16, 1056–1065.

Fletcher, T.L., Cameron, P., DeCamilli, P. and Banker, G. (1991) The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. J. Neurosci. 11, 1617–1626.

Fraichard, A., Chassande, O., Bilbaut, G., Dehay, C., Savatier, P. and Samarut, J. (1995) In vitro differentiation of embryonic stem cells into glial cells and functional neurons. J. Cell Science 108, 3181–3188.

Frederiksen, K. and McKay, R.D.G. (1988) Proliferation and differentiation of rat neuroepithelial precursor cells in vivo. J. Neurosci. 8, 1144–1151.

Gensburger, C., Labourdette, G. and Sensenbrenner, M. (1987) Brain basic fibroblast growth factor stimulates the proliferation of rat neuronal precursor cells in vitro. FEBS Lett. 217, 1–5.

Ghosh, A. and Greenberg, M.E. (1995) Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron 15, 89–103.

Ginty, D.D., Kornhauser, J.M., Thompson, M.A., Bading, H., Mayo, K.E., Takahashi, J.S. and Greenberg, M.E. (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science 260, 238–241.

Joyner, A.L. and Martin, G.R. (1987) En-1 and En-2, two mouse genes with sequence homology to the *Drosophila* engrailed gene: expression during embryogenesis. Genes Dev. 1, 29–38.

Kemler, R., Brulet, P., Schnebelen, M.-T., Gaillard, J. and Jacob, F. (1981) Reactivity of monoclonal antibodies against intermediate filament proteins during embryonic development. J. Embryol. Exp. Morphol. 64, 45–60.

Kilpatrick, T.J. and Bartlet, P.F. (1993) Cloning and growth of multipotential neuronal precursors: requirements for proliferation and differentiation. Neuron 10, 255–265.

Kimelberg, H.K., Narumi, S. and Bourke, R.S. (1978) Enzymatic and

morphologic properties of primary rat brain astrocyte cultures and enzymatic development in vivo. Brain Res. 153, 55–77.

Kimelman, D. and Kirschner, M. (1987) Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early *Xenopus* embryo. Cell *51*, 869–877.

Kurtz, A., Zimmer, A., Schnutgen, F., Bruhing, G., Spener, F. and Müller, T. (1994) The expression pattern of a novel gene encoding brain-fatty acid binding protein correlates with neuronal and glial cell development. Development 120, 2637–2649.

Lee, K.-F., Li, E., Huber, J., Landis, S., Sharpe, A.H., Chao, M.V. and Jaenisch, R. (1992) Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell 69, 737–749.

Lendahl, U., Zimmerman, L.B. and McKay, R.D.G. (1990) CNS stem cells express a new class of intermediate filament protein. Cell 60, 585–595.

Mahon, K.A., Westphal, H. and Gruss, P. (1988) Expression of homeobox gene Hox 1.1 during mouse embryogenesis. Development 104 (Suppl.), 187–195.

Martin, G.R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA 78, 7634–7638.

Mortensen, R.M., Conner, D.A., Chao, S., Geisterfer-Lowrance, A.A.T. and Seidman, J.G. (1992) Production of homozygous mutant ES cells with a single targeting construct. Mol. Cell Biol 12, 2391–2395.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. and Roder, J.C. (1993) Derivation of completely cell-culture-derived mice from early -passage embryonic stem cells. Proc. Natl. Acad. Sci. USA 90, 8424–8428.

Ray, J., Peterson, D.A., Schinstine, M. and Gage, F.H. (1993) Proliferation, differentiation and long-term culture of primary hippocampal neurons. Proc. Natl. Acad. Sci. USA 90, 3602–3606.

Rizzino, A. and Growley, C. (1980) Growth and differentiation of embryonal cartinoma cell line F9 in defined media. Proc. Natl. Acad. Sci. USA 77, 457–461.

Robertson, E.J. (1987) Embryo-derived stem cell lines. In Robertson, E.J. (ed.), Teratocarcinoma and Embryonic Stem Cells: a Practical Approach, IRL Press, Washington, D.C., pp. 71–112.

Segal, M. (1983) Rat hippocampal neurons in culture: responses to electrical and chemical stimuli. J Neurophysiol 50, 1249–64.

Segal, M. and Barker, J.L. (1984) Rat hippocampal neurons in culture: potassium conductances. J Neurophysiol 51, 1409–33.

Shaw, G., Banker, G.A. and Weber, K. (1985) An immunofluorescence study of neurofilament protein expression by developing hippocampal neurons in tissue culture. Eur. J. Cell Biol. 39, 205–216.

Simeone, A., Acampora, D., Arcioni, L., Andrews, P.W., Boncinelli, E. and Mavilio, F. (1990) Sequential activation of HOX2 homeobox genes by retinoic acid in human embryonal carcinoma cells. Nature 346, 763–766.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A. and Boncinelli, E. (1992) Nested expression domains of four homeobox genes in developing rostral brain. Nature 358, 687–690.

Sommer, I. and Schachner, M. (1981) Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. Dev. Biol. 83, 311–27.

Stemple, D.L. and Anderson, D.J. (1992) Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell 71, 973–985.

Swiatek, P.J. and Gridley, T. (1993) Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene *Krox20*. Genes Dev. 7, 2071–2084.

Vicario-Abejon, C., Johe, K.K., Hazel, T.G., Collazo, D. and McKay, R.D.G. (1995) Functions of basic-fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. Neuron 15, 105–114.

Yamaguchi, T., Harpal, K., Henkemeyer, M. and Rossant, J. (1994) fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. Genes Dev. 8, 3032–3044.

Zackenfels, K., Oppenheim, R.W. and Rohrer, H. (1995) Evidence for an important role of IGF-1 and IGF-II for the early development of chick sympathetic neurons. Neuron 14, 731–41.

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 14809–14814, December 1997
Neurobiology

In vitro-generated neural precursors participate in mammalian brain development

OLIVER BRÜSTLE*, A. CYRIL SPIRO, KHALAD KARRAM*, KHALID CHOUDHARY, SHIGEO OKABE†, AND RONALD D. G. MCKAY‡

Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892-4092

Edited by Pasko Rakic, Yale University School of Medicine, New Haven, CT, and approved October 23, 1997 (received for review June 11, 1997)

ABSTRACT **During embryogenesis, pluripotent stem cells segregate into daughter lineages of progressively restricted developmental potential. *In vitro*, this process has been mimicked by the controlled differentiation of embryonic stem cells into neural precursors. To explore the developmental potential of these cell-culture-derived precursors *in vivo*, we have implanted them into the ventricles of embryonic rats. The transplanted cells formed intraventricular neuroepithelial structures and migrated in large numbers into the brain tissue. Embryonic-stem-cell-derived neurons, astrocytes, and oligodendrocytes incorporated into telencephalic, diencephalic, and mesencephalic regions and assumed phenotypes indistinguishable from neighboring host cells. These observations indicate that entirely *in vitro*-generated neural precursors are able to respond to environmental signals guiding cell migration and differentiation and have the potential to reconstitute neuronal and glial lineages in the central nervous system.**

The ability to isolate, proliferate, and genetically manipulate embryonic stem (ES) cells is one of the major achievements in experimental biology (1, 2). The totipotency of these cells has kindled numerous efforts to generate tissue-specific precursors from ES cells *in vitro*. The controlled differentiation of ES cells into stem cells of defined lineages provides experimental access to early embryonic development and may eventually lead to alternative donor sources for tissue reconstruction. A central question concerning the biology of ES-cell-derived precursors is to what extent these cells resemble their *in vivo* counterparts. Are precursor cells generated outside the context of a multicellular organism sufficiently responsive to positional cues to participate in the development and histogenesis of a living host? Hematopoietic stem cells derived from ES cells have, indeed, been shown to reconstitute the lymphoid, myeloid, and erythroid lineages after transplantation into irradiated mice (3). During the last 3 years, several groups have demonstrated that mature neurons and glia can be derived from ES cells *in vitro* (4–7). Recently, we have reported an efficient procedure for the generation of proliferative neural precursors from ES cells. *In vitro*, these precursors generate functional neurons, astrocytes, and oligodendrocytes (8). *In vivo* reconstitution would be a more stringent test of the potential of ES cells to acquire central nervous system fates. The limited self-renewal in the adult mammalian brain precludes the kind of ablation and reconstitution experiments used to study *in vivo* differentiation of hematopoietic progenitors. We have, therefore, used a different approach and introduced ES-cell-derived neural precursors into the developing brain. Previous studies have revealed that primary neuroepithelial precursors implanted into the ventricle of embryonic rats incorporate extensively into the host brain and undergo region-specific differentiation into neurons and glia (9–11). Herein, we show that ES-cell-derived neural precursors grafted into the embryonic ventricle migrate into the host brain and contribute to all three principal lineages of the nervous system. Our results suggest that neuroepithelial precursors derived from ES cells in the absence of positional cues can migrate and differentiate according to local signals in the host environment.

MATERIALS AND METHODS

ES Cell Culture. ES cells (line J1; ref. 12) were maintained on γ-irradiated fibroblasts in DMEM containing 20% fetal calf serum, 0.1 mM 2-mercaptoethanol, nucleosides, nonessential amino acids, and human recombinant leukemia inhibitory factor (1000 units/ml). Cells were passaged once onto gelatin-coated dishes and then aggregated to form embryoid bodies in bacterial dishes in the absence of leukemia inhibitory factor. Four-day-old embryoid bodies were plated in tissue culture dishes and propagated for 5–12 days in ITSFn medium (DMEM/F-12 containing insulin at 5 μg/ml, transferrin at 50 μg/ml, 30 nM selenium chloride, and fibronectin at 5 μg/ml; ref. 8).

Intrauterine Transplantation. Cells were trypsinized and triturated to single-cell suspensions in the presence of 0.1% DNase. Timed-pregnant Sprague–Dawley rats were anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg), and 0.1–1 × 10^6 cells were injected into the telencephalic vesicle of each embryo as described (11).

Immunohistochemistry. Zero to 15 days after spontaneous birth, recipients were anesthetized and perfused with 4% paraformaldhyde in PBS (stillborn recipients were fixed by immersion). Serial 50-μm Vibratome sections were characterized with antibodies to microtubule-associated protein 2 (Sigma, dilution, 1:200), nestin (dilution, 1:1,000), glial fibrillary acidic protein (GFAP; ICN, dilution, 1:100), (CNPase; Sigma, dilution, 1:200), neurofilament (SMI311, Sternberger Monoclonals, Baltimore, MD; dilution, 1:500), NeuN (Chemicon, dilution, 1:50), tyrosine hydroxylase (Eugene Tech Intl., Ridgefield Park, NJ; dilution, 1:200), M2 and M6 (refs. 13 and 14; dilution, 1:10). Antigens were visualized by using appropriate fluorophore- or peroxidase-conjugated secondary antibodies. To assay alkaline phosphatase activity, sections were incubated at 37°C in 100 mM Tris·HCl, pH 9.5/100 mM

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/97/9414809-6$0.00/0
PNAS is available online at http://www.pnas.org.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: E, embryonic day; ES cell, embryonic stem cell; P, postnatal day; GFAP, glial fibrillary acidic protein; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase.

*Present address: Institute of Neuropathology, University of Bonn Medical Center, 53105 Bonn, Germany.
†Present address: National Institute of Bioscience and Human-Technology, 1–1 Higashi, Tsukuba Ibaraki, 305, Japan.
‡To whom reprint requests should be addressed at: Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Building 36, Room 5A29, 36 Convent Drive, Bethesda, MD 20892-4092.

NaCl/50 mM $MgCl_2$/nitroblue tetrazolium at 0.3 mg/ml/5-bromo-4-chloro-3-indolyl phosphate at 0.175 mg/ml. After 3–10 min, the staining was stopped by transferring the sections to 10 mM Tris·HCl/1 mM EDTA. Specimens were examined on Zeiss Axioplan, Axiovert, and Laser Scan microscopes.

***In Situ* Hybridization.** Donor cells were identified by using a digoxigenin-end-labeled oligonucleotide probe to the mouse major satellite (15). DNA–DNA *in situ* hybridization was performed as described (11). Briefly, sections were treated with Pronase at 25 μg/ml in 2× SSC/5 mM EDTA for 15 min at 37°C, dehydrated, and denatured in 70% formamide/2× SSC at 85°C for 12 min. After dehydration in ice-cold ethanols, sections were hybridized overnight at 37°C in 65% formamide/2× SSC/salmon sperm DNA at 250 μg/ml. Washes were 50% formamide/2× SSC (30 min, 37°C) and 0.5× SSC (37°C, 15 min). Hybridized probe was detected by using alkaline phosphatase or fluorophore-conjugated antibodies to digoxigenin (Boehringer).

RESULTS

Widespread Incorporation of ES-Cell-Derived Neural Precursors into the Developing Brain. For transplantation, 4-day embryoid bodies were plated on tissue culture dishes and grown in ITSFn medium. This medium has previously been shown to strongly select for neural precursors (8). During the first 72 h in ITSFn, a large proportion of the cells died. Most of the remaining cells acquired an elongated phenotype strongly reminiscent of neuroepithelial precursor cells. These cells also expressed nestin, an intermediate filament typically present in neural precursor cells (16). After 6 days in ITSFn medium, typically more than 80% of the cells were nestin-positive. The remaining cells showed varied morphologies with focal expression of SSEA-1 and keratin 8, i.e., antigens typically expressed in undifferentiated embryonic tissues and primitive ectoderm (refs. 17 and 18; data not shown). After 5–12 days in ITSFn, cells were harvested and used for transplantation. Recipient animals were grafted between embryonic day (E) 16 and E18 and sacrificed between postnatal day (P) 0 and P15. Clusters of donor cells were detected in the ventricles of all successfully injected pups (see below). ***In situ*** hybridization revealed that large numbers of mouse cells left the ventricle and migrated into various host brain regions, including cortex, striatum, septum, thalamus, hypothalamus, and tectum (Figs. 1 and 2 *A*, *E*, *F*, and *K* and Table 1). The transplanted cells integrated individually into the host tissue and were only detectable by virtue of their genetic difference. The number of incorporated cells varied considerably among individual recipients and brain regions. Quantitative stereology and a detailed assessment of donor cell survival and proliferation will be required to assess what proportion of the transplanted cells integrates into the host brain. Preliminary cell counts have revealed up to 650 incorporated cells in single coronal 50-μm sections.

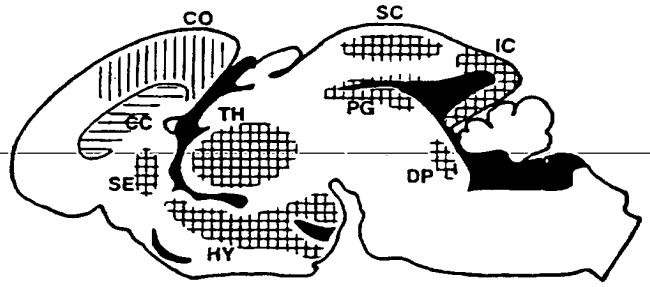


FIG. 1. Distribution of ES-cell-derived neural precursors after implantation into the telencephalic vesicle of E16–E18 rats. The schematic represents a midsagittal section through the brain of a newborn recipient. After leaving the ventricular system (solid areas), neurons (vertical lines) and astrocytes (horizontal lines) occupy overlying territories. Donor-derived neurons integrate preferentially into gray matter regions exhibiting neurogenesis until or beyond the time of implantation. ES-cell-derived astrocytes also incorporate into white matter regions such as the corpus callosum (CC). CO, cortex; DP, dorsal pontine area; HY, hypothalamus; IC, inferior colliculus; PG, periaqueductal gray; SC, superior colliculus; SE, septum; TH, thalamus. Donor-derived neurons and astrocytes were also detected in hippocampus (Fig. 3*B*), olfactory bulb (Fig. 4 *A* and *B*), and striatum (Fig. 2*E*).

Differentiation into Neurons and Glia. The differentiation of the incorporated cells was assessed by using antibodies to cell-type-specific antigens in conjunction with a mouse-specific DNA probe or mouse-specific antibodies to M6 and M2 (13, 14). Hybridized neurons expressing the neuronal antigens NeuN (19) and microtubule-associated protein 2 were detected at tel-, di-, and mesencephalic levels (Fig. 2 *I* and *L*). The shape, size, and orientation of these cells were indistinguishable from adjacent host neurons (Fig. 2*I*). Confocal laser microscopy allowed detailed reconstruction of individual phenotypes. ES-cell-derived neurons exhibited characteristic polar morphologies with segregation of neurites into dendrites and axons (Fig. 2 *B–D* and *G*). Both classes of neurites frequently extended several hundred micrometers into the adjacent host neuropil (Fig. 2 *G* and *H*). Donor-derived neurons were readily detectable at birth but appeared to undergo further morphological maturation in the postnatal period. The example in Fig. 2*M* shows an ES-cell-derived neuron with prominent dendritic spines in the thalamus of a 2-week-old host. Neurons integrating into the host cortex frequently displayed morphologies of projection neurons with long apical dendrites reaching into the superficial cortical layers and basal axons extending several hundred micrometers into the corpus callosum (Fig. 2 *B–D*). An example of a ES-cell-derived pyramidal neuron in the cortex of a neonatal host is shown in Fig. 2*D*. The donor neurons generated an extensive axonal network throughout the host gray and white matter, reaching from the most rostral regions such as the olfactory bulb to the brainstem (Fig. 3). Within the white matter, donor-derived axons frequently assembled into prominent fiber bundles running alongside host axons through the major axonal trajectories, including the corpus callosum (Fig. 3*A*), anterior commissure, striatal fiber bundles (Fig. 3*C*), and various other endogenous fiber tracts. Gray matter regions exhibiting dense donor-derived axonal networks included cortex (Fig. 3*A*), hippocampus (Fig. 3*B*), septum, striatum, thalamus (Fig. 3*D*), hypothalamus, tegmentum, tectum, and brainstem.

Astrocytes generated by the transplanted ES cells were found in a distribution similar to that of donor-derived neurons. The most prominent accumulations were detected in the ventral diencephalon and in tectum. In addition, these cells efficiently incorporated into white matter regions such as the corpus callosum (Fig. 1). Donor-derived astrocytes strongly expressed M2, a species-specific antigen frequently used for the identification of mouse astrocytes in xenografts (Fig. 4 *A* and *B* and ref. 21). Their astroglial identity was confirmed by double labeling with an antibody to GFAP of cells labeled with either the mouse satellite probe or the M2 antibody (Fig. 4*B*). Encountered only occasionally in newborn recipients, these cells were more frequently detected in animals sacrificed at P15 (Table 1). ES-cell-derived astrocytes were morphologically indistinguishable from their host counterparts, and GFAP immunofluorescence showed no differences in size or branching pattern between the two populations. Typical for astroglia, donor-derived astrocytes often extended processes to adjacent blood vessels.

In addition to neurons and astrocytes, donor-derived oligodendrocytes were found in the transplanted rat brains. The identity of these cells was confirmed by *in situ* hybridization with the mouse satellite probe and subsequent immunohisto-

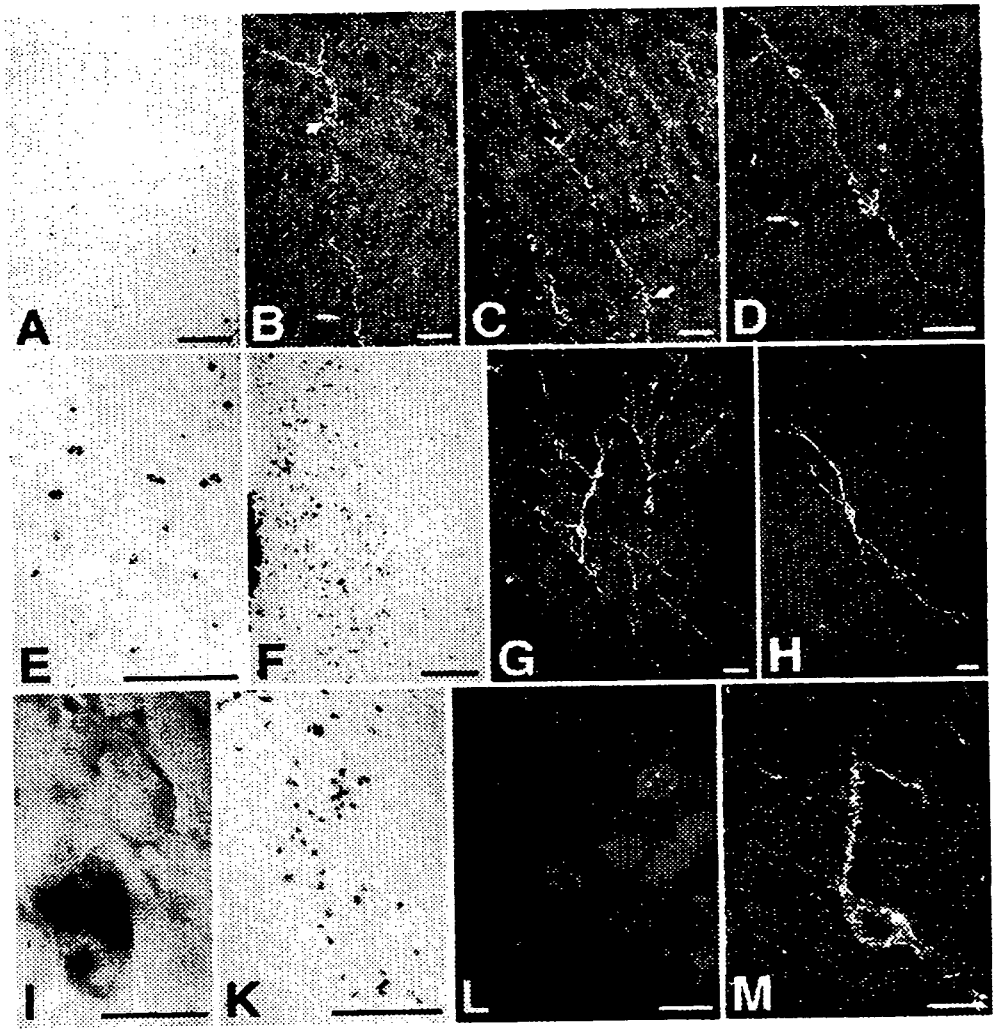


FIG. 2. ES-cell-derived neural precursors injected into the telencephalic vesicle of fetal rats incorporate individually into a variety of host brain regions and differentiate into neurons. Donor cells are identified by *in situ* hybridization using a digoxigenin-labeled probe to mouse satellite DNA (*A*, *E*, *F*, and *I–L*). Immunofluorescence detection of the mouse-specific antigen M6 and confocal laser microscopy were used to reconstruct individual neuronal profiles (*B–D*, *G*, *H*, and *M*). (*A–D*) Six days after injection into the telencephalic vesicle of an E17 rat, donor cells have left the ventricle and incorporated into the host cortex. The ES-cell-derived neurons show prominent apical dendrites and basal axons entering the corpus callosum, a morphology appropriate for cortical projection neurons (arrows: perikaryon). Note the characteristic pyramidal morphology in *D*. (*E*) Incorporated donor cells in the striatum of a 2-week-old rat transplanted at E18. (*F–H*) Donor-derived cells in the host hypothalamus. In contrast to cortex, neurons incorporating into the diencephalon frequently exhibited multipolar morphologies (*G* and *H*). (*I*) Host- and donor-derived neurons in the septum of a newborn rat. Both neurons show expression of microtubule-associated protein 2; the donor-derived cell is identified by *in situ* hybridization. (*K–M*) Incorporated cells in the host thalamus of newborn (*K*) and 2-week-old rats (*L* and *M*). In *L*, ES-cell-derived neurons are visualized by fluorescence *in situ* hybridization (green dots) and subsequent immunofluorescence analysis with an antibody to the nuclear neuronal antigen NeuN (red). Note the mature neuronal phenotype of the integrated cells with the presence of dendritic spines (*M*). [Bars = 100 μm (*A*, *E*, *F*, and *K*), 20 μm (*B–D*, *G*, *H*, *L*, and *M*), and 10 μm (*I*).]

chemical detection of CNPase, a marker for myelin and oligodendroglia (22). ES-cell-derived CNPase-positive oligodendrocytes were only detected in the brains of 2-week survivors and their distribution was restricted to white matter regions such as the corpus callosum and striatal fiber tracts. Size, orientation, and CNPase expression of these cells were indistinguishable from their host counterparts (Fig. 4 *C* and *D*).

Formation of Mitotic Neuroepithelium. Cells remaining in the host ventricle formed large clusters containing prominent neuroepithelial formations. These formations were particularly prominent in the 2-week survivors and—upon cross-sectioning—closely resembled tightly packed neural tubes (Fig. 5*A*). Individual tubes consisted of columnar epithelium with high mitotic activity at the luminal surface (Fig. 5*A Inset*). The epithelial cells showed strong expression of nestin (Fig. 5 *B* and *C*) and brain fatty-acid-binding protein (data not shown), antigens typically expressed in the early neuroepithelium (16, 23, 24). Cells expressing neuronal antigens, including microtubule-associated protein 2, neurofilament, and tyrosine hydroxylase, were restricted to the periphery of the formations, suggesting an inside-out gradient of differentiation (Fig. 5*D*). Thus, by morphology, antigen expression, and gradients of proliferation and differentiation, these structures are very similar to the developing neuroepithelium. In addition, intraventricular grafts contained small clusters of still undifferentiated embryonic cells. These cells were easily recognized by their expression of alkaline phosphatase, an enzyme typically present in undifferentiated embryonic cells (25). Whereas clusters of alkaline phosphatase-positive cells were frequently

Table 1. Differentiation of ES-cell-derived neural precursors after transplantation into the embryonic rat brain

	Parenchyma		Ventricle		
Age at analysis	Neurons	Glia	NEF	AP	NNT
P0	TDM	D	+	+	−
P0	TDM	T	−	+	−
P0	TD	−	+	−	−
P0	TM	−	−	+	−
P0	TDM	T	−	++	−
P0	D	D	+	+	−
P1	TDM	−	−	++	−
P1	TD	−	−	++	−
P1	TDM	−	−	+	−
P1	TD	−	−	+	−
P1	TDM	TDM	−	+	−
P1	TDM	−	−	+	−
P1	TDM	D	−	+	−
P1	TDM	TM	−	++	−
P15	DM	DM	+	+	+
P15	TDM	TD	−	−	+
P15	TDM	TDM	+	−	+
P15	TM	TM	+	+	+
P15	T	TD	+	−	+
P15	TDM	TM	+	−	+
P15	TD	TD	+	+	+

ES cells (line J1) aggregated to embryoid bodies and grown for 5–12 days in ITSFn medium were injected into the ventricle of E16–E18 rats. Recipients were sacrificed between P0 and P15 and donor-derived neurons were identified by DNA *in situ* hybridization in conjunction with immunohistochemical detection of NeuN or by expression of M6 and unequivocal morphological criteria (presence of dendrites and axons). ES-cell-derived astrocytes were detected with an antibody to M2 or by DNA *in situ* hybridization in conjunction with immunohistochemical detection of GFAP. Intraventricular donor cell clusters were assayed for the presence of nestin-positive neuroepithelial formations (NEF), clusters of undifferentiated, alkaline phosphatase-positive cells (AP; ++, numerous clusters; +, occasional clusters), and differentiated nonneural tissue (NNT). Each row represents one recipient animal. The integration patterns show considerable interindividual variability. There is an increase of glial cells and a decrease of AP-positive cells with increasing survival time. T, telencephalon; D, diencephalon; M, mesencephalon.

encountered in newborn animals, they were only occasionally detected in the 2-week survivors, arguing for a gradual differentiation of the embryonic cells. Disappearance of the alkaline phosphatase-positive cells was inversely correlated with the emergence of nonneural tissue in the P15 animals, where occasional islands containing adenoid structures, cartilage, and epidermis were found within the intraventricular clusters (Table 1). These observations indicate that undifferentiated embryonic cells present in the transplanted cell suspension form intraventricular teratomas.

DISCUSSION

Our results demonstrate that ES-cell-derived neural precursors transplanted into the developing mammalian brain are able to generate neuronal and glial lineages. Upon injection into the telencephalic vesicle of fetal rats, the donor cells leave the ventricle and migrate into the recipient brain, where they differentiate into neurons, astrocytes, and oligodendrocytes. Incorporation of the transplanted cells is not random but occurs in patterns compatible with the host brain development. The donor cells integrated preferentially into cortex, hippocampus, striatum, septum, the medial diencephalic nuclei, and tectum. These regions are known to continue neurogenesis until or even beyond the time of transplantation (26). Temporally, neurons, astrocytes, and oligodendrocytes appeared successively. Few astrocytes were present neonatally when

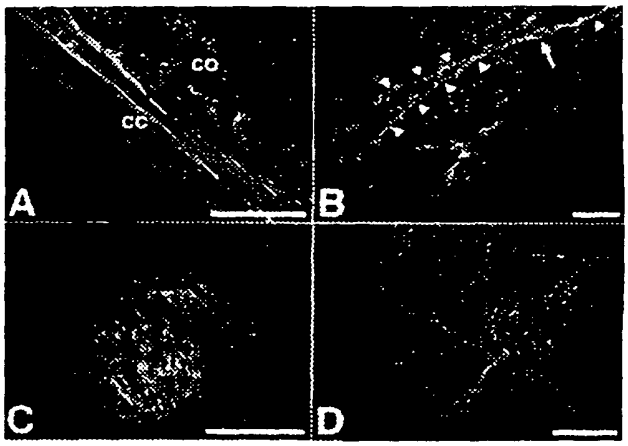


FIG. 3. Extensive axonal innervation of the host brain. The ES-cell-derived neurons generated a dense axonal network within the recipient brains. Abundant M6-positive axons were found at all levels in both gray and white matter. (*A*) Donor-derived axons in corpus callosum (cc) and deep layer cortex (co) of a 2-week-old recipient. (*B*) Axonal innervation of the hippocampal stratum oriens. The M6 immunofluorescence also depicts the perikaryon (arrow) and dendrites (arrowheads) of a large horizontal neuron in the upper stratum oriens. The morphology of this cell is very similar to the outline of Golgi-impregnated horizontal neurons in this area (20). (*C*) Abundant donor-derived axons in a striatal fiber tract of 2-week-old recipient brain. (*D*) ES-cell-derived axons in the thalamus of a newborn recipient transplanted at E17. (Bars = 50 μm.)

compared with 2-week-old recipients (Table 1), and oligodendrocytes were only detected in the P15 animals. This delayed appearance of glial cells corresponds well with the timing of host gliogenesis that has been shown to be primarily a postnatal event (27). In addition, neurons and glia showed differences in their distribution. Although neurons incorporated preferentially into gray matter regions, astrocytes invaded both gray and white matter, and oligodendrocytes were found only in fiber tracts. These observations indicate that neural precursors

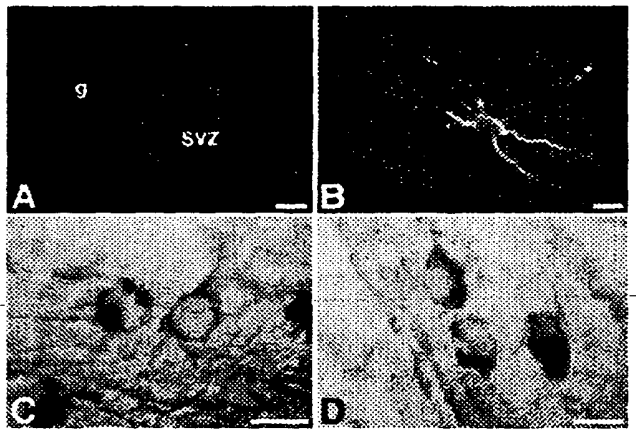


FIG. 4. Incorporation of ES-cell-derived glia. (*A* and *B*) ES-cell-derived astrocytes have migrated into the granular layer (g) of the olfactory bulb of a 2-week-old host. Cells are visualized with an antibody to the mouse-specific antigen M2 (red). SVZ, olfactory subventricular zone. An individual astrocyte, double labeled with an antibody to GFAP (green), is shown in *B*. (*C* and *D*) ES-cell-derived oligodendrocytes in the rostral (*C*) and caudal (*D*) corpus callosum of a 2-week-old host brain. The donor cells, identified by DNA *in situ* hybridization (black), are morphologically indistinguishable from adjacent host oligodendrocytes. Host- and donor-derived oligodendrocytes exhibit equivalent immunoreactivity to CNPase (red). [Bars = 100 μm (*A*) and 10 μm (*B–D*).]

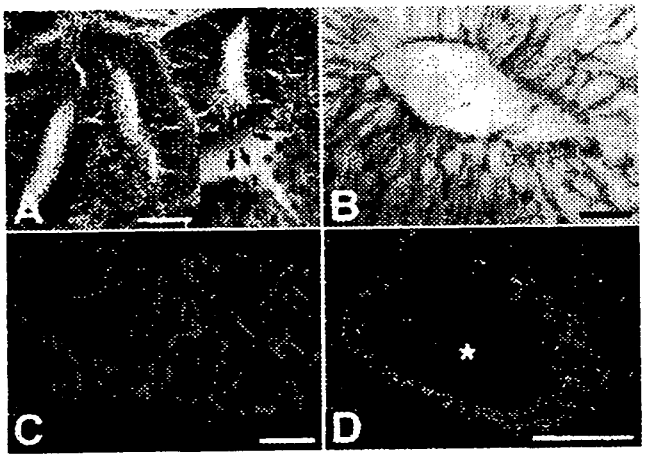


FIG. 5. Generation of neuroepithelial formations. (*A* and *B*) Eight days after intrauterine transplantation, the donor cells have generated numerous neural tube-like structures within the host ventricle. Like the developing neural tube, these structures exhibit high mitotic activity at the luminal surface (*A*) (hematoxylin/eosin; arrows in *Inset* indicate mitotic figures) and strong expression of the intermediate filament nestin (*B*). (*C* and *D*) Neuroepithelial formation in the ventricle of a 2-week-old animal transplanted at E18. The formation contains abundant radially oriented nestin-positive processes (*C*). As in the early neuroepithelium, there is an inside-out gradient of differentiation with neuronal markers being expressed at the periphery of the formation (*D*) (green, tyrosine hydroxylase; red, M6; *, center of formation). [Bars = 100 μm (*A* and *D*) and 20 μm (*B* and *C*).]

derived from ES cells and transplanted into the mammalian brain are susceptible to environmental cues guiding cell fate determination and differential migration. Furthermore, donor-derived neurons appear to respond to local differentiation cues. This is particularly evident in cortex, where they acquired morphological features characteristic for cortical projection neurons, including a pyramidal cell body, long apical dendrites, and basal axons projecting into the corpus callosum (Fig. 2 *B–D*). The innervation of the host brain by the transplanted cells further suggests that donor-derived neurons remain responsive to host-mediated axon guidance. Detectable at all levels of the recipient brain, donor-derived axons bundled and extended through the major host trajectories, running alongside endogenous axons and obeying the border of the fiber tracts (Fig. 3*C*). Donor-derived astrocytes and oligodendrocytes were similarly indistinguishable from their endogenous counterparts and only detectable by virtue of their genetic difference. Donor and host glia exhibited equivalent expression of the cell-type-specific antigens GFAP and CNPase. Many of the ES-cell-derived astrocytes assumed perivascular locations, with one or several processes extending to the capillary wall—a feature typically observed in astrocytes involved in blood–brain barrier formation (data not shown).

The migration and differentiation patterns of the transplanted ES cells are in accordance with those obtained after intrauterine transplantation of primary cells derived from the embryonic brain (9–11). In both cases, the donor cells incorporate preferentially into regions exhibiting protracted neurogenesis until late gestation and adopt local phenotypic features. These observations indicate that primary neuroepithelial cells and neural precursors derived from ES cells respond very similarly to environmental cues. Our data strongly suggest that neuroepithelial cells generated *in vitro* from ES cells can act as neural precursors *in vivo* and contribute neurons and glia to the developing mammalian brain. These findings significantly extend results of previous studies showing that retinoic acid-induced ES cells exhibit antigenic and electrophysiological properties of neurons and glia *in vitro* (4–7) and that the differentiated phenotype of retinoic acid-induced ES or teratocarcinoma cells can be maintained after transplantation into the adult brain (28–32). In contrast to these studies, we were less interested in the forceful induction and subsequent maintenance of a neuronal phenotype but rather in the interaction of still undifferentiated neural precursors with the developing brain. The widespread neuronal and glial integration obtained after transplantation of ES-cell-derived precursors provides an impressive example for the dominant role of non-cell-autonomous signals during neural migration and differentiation. The fact that a cell never previously exposed to a nervous system is able to migrate into cortex and to differentiate into an appropriate local phenotype illustrates that cell communities in individual brain regions harbor sufficient cues to maintain their local identity and to foster their own development through precursor cell recruitment.

It is a well-described phenomenon that ES cells transplanted to an adult host frequently develop teratomas and teratocarcinomas (for review, see ref. 33). In striking contrast, both ES and teratocarcinoma cells have been shown to participate in normal development upon introduction into early embryos at the blastocyst stage (for review, see ref. 34). Our observations, along with similar studies on ES-cell-derived hematopoietic progenitors (3), provide an interesting intermediate between these two scenarios. They show that the ability to participate in normal development is not restricted to undifferentiated ES cells but extends to their more differentiated progeny. Neural and hematopoietic precursors derived from ES cells *in vitro* are able to reconstitute neural and hematopoietic lineages after transplantation into living hosts. It is likely that such a contribution to host tissue formation requires close physical contact between the transplanted cells and the target tissue. One explanation for the conspicuous formation of neural tube-like structures within the host ventricles might be that ES cells differentiated into neural precursors but physically separated from the brain tissue are not sufficiently exposed to local cues mediating precursor cell recruitment and thus develop autonomously into primitive nervous system tissue. ES cells not sufficiently differentiated into neural precursors might evade recruitment because they are unable to respond to tissue-specific guidance cues. Because of their pluripotency, these cells can develop into a variety of tissues. Islands of nonneural tissue observed within the intraventricular clusters are most likely derivatives of still undifferentiated ES cells present in the transplanted cell suspension.

Although our results outline the developmental potential of *in vitro*-generated neural precursors at a basic neurobiological level, the *in vivo* reconstitution of neuronal and glial lineages by transplanted ES-cell-derived donor cells might eventually be exploited for cell replacement strategies. This idea receives strong impetus from recent findings implying that embryonic stem cells can be obtained from adult tissue by transferring nuclei of differentiated cells into oocytes (35), a perspective offering the possibility to generate virtually unlimited numbers of tissue-specific and genetically modified donor cells from the same individual. Transplant experiments in rodents further indicate that the adult brain may retain some of the cues required for regional cellular differentiation (36–38). These observations would suggest that region-specific differentiation of transplanted ES-cell-derived precursors is not limited to the developing nervous system. The feasibility of ES-cell-based replacement strategies will critically depend on the ability to generate highly purified donor populations susceptible to host regulation.

The possibility of introducing ES-cell-derived neurons and glia into the developing nervous system also offers an exciting approach for the study of neurological mutants. The rapid proliferation of ES cells and their susceptibility to genetic

manipulation allows the generation of large numbers of genetically modified donor cells. The properties of these cells can then be assayed *in vivo* in a wild-type recipient brain. The data presented herein show that the full range of neuronal and glial phenotypes might be accessible with this approach. Analysis is not restricted to cell migration and differentiation but may include aspects such as axon outgrowth and guidance, myelination, and the susceptibility of distinct genotypes to degenerative and neoplastic brain disease. Transplantation of ES-cell-derived neural precursors should be especially useful for the analysis of targeted gene deletions. Homozygous knockout ES cells from mutants exhibiting very early embryonic lethality can be differentiated *in vitro* and their neural offspring can be analyzed in the context of a developing brain. Incorporation of ES-cell-derived precursors into the central nervous system also offers the possibility to analyze the neural phenotype of null mutants without prior generation of a knockout animal.

We thank Carl Lagenaur for providing the M6 and M2 antibodies. We gratefully acknowledge Kim Jones and Robert Green for help with the tissue processing, Carolyn Smith for her advice on confocal laser microscopy, and Tom Hazel for his helpful suggestions.

1. Evans, M. J. & Kaufman, M. H. (1981) *Nature (London)* **292,** 154–156.
2. Martin, G. R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7634–7638.
3. Palacios, R., Golunski, E. & Samaridis, J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7530–7534.
4. Bain, G., Kitchens, D., Yao, M., Huettner, J. E. & Gottlieb, D. I. (1995) *Dev. Biol.* **168,** 342–357.
5. Fraichard, A., Chassande, O., Bilbaut, G., Dehay, C., Savatier, P. & Samarut, J. (1995) *J. Cell Sci.* **108,** 3181–3188.
6. Strübing, C., Ahnert-Hilger, G., Shan, J., Wiedenmann, B., Hescheler, J. & Wobus, A. M. (1995) *Mech. Dev.* **53,** 275–287.
7. Finley, M. F. A., Kulkarni, N. & Huettner, J. E. (1996) *J. Neurosci.* **16,** 1056–1065.
8. Okabe, S., Forsberg-Nilsson, K., Spiro, A. C., Segal, M. & McKay, R. D. G. (1996) *Mech. Dev.* **59,** 89–102.
9. Fishell, G. (1995) *Development (Cambridge, U.K.)* **121,** 803–812.
10. Campbell, K., Olsson, M. & Björklund, A. (1995) *Neuron* **15,** 1259–1273.
11. Brüstle, O., Maskos, U. & McKay, R. D. G. (1995) *Neuron* **15,** 1275–1285.
12. Li, E., Bestor, T. H. & Jaenisch, R. (1992) *Cell* **69,** 915–926.
13. Lagenaur, C. & Schachner, M. (1981) *J. Supramol. Struct. Cell. Biochem.* **15,** 335–346.
14. Lund, R. D., Chang, F. L. F., Hankin, M. H. & Lagenaur, C. F. (1985) *Neurosci. Lett.* **61,** 221–226.
15. Hörz, W. & Altenburger, W. (1981) *Nucleic Acids Res.* **9,** 683–696.
16. Lendahl, U., Zimmermann, L. B. & McKay, R. D. G. (1990) *Cell* **60,** 585–595.
17. Solter, D. & Knowles, B. B. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5565–5569.
18. Kemler, R., Brület, P., Schnebelen, M. T., Gaillard, J. & Jacob, F. (1981) *J. Embryol. Exp. Morphol.* **64,** 45–60.
19. Mullen, R. J., Buck, C. R. & Smith, A. M. (1992) *Development (Cambridge, U.K.)* **116,** 201–211.
20. de Lorente, N.-R. (1934) *J. Psychol. Neurol.* **46,** 113–177.
21. Zhou, H. F., Lee, L. H. C. & Lund, R. D. (1990) *J. Comp. Neurol.* **292,** 320–330.
22. Sprinkle, T. J. (1989) *CRC Crit. Rev. Neurobiol.* **4,** 235–301.
23. Frederiksen, K. & McKay, R. D. G. (1988) *J. Neurosci.* **8,** 1144–1151.
24. Kurtz, A., Zimmer, A., Schnütgen, F., Brüning, G., Spener, F. & Müller, T. (1994) *Development (Cambridge, U.K.)* **120,** 2637–2649.
25. Bernstine, E. G., Hooper, M. L., Grandchamp, S. & Ephrussi, B. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 3899–3903.
26. Altman, J. & Bayer, S. A. (1995) *Atlas of Prenatal Rat Brain Development* (CRC, Boca Raton, FL).
27. Levison, S. W., Chuang, C., Abramson, B. J. & Goldman, J. E. (1993) *Development (Cambridge, U.K.)* **119,** 611–622.
28. Trojanowski, J. Q., Mantione, J. R., Lee, J. H., Seid, D. P., You, T., Inge, L. J. & Lee, V. M. Y. (1993) *Exp. Neurol.* **122,** 283–294.
29. Wojcik, B. E., Nothias, F., Lazar, M., Jouin, H., Nicolas, J. F. & Peschanski, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 1305–1309.
30. Morassutti, D. J., Staines, W. A., Magnuson, D. S. K., Marshall, K. C. & McBurney, M. W. (1994) *Neuroscience* **58,** 753–763.
31. Magnuson, D. S., Morassutti, D. J., Staines, W. A., McBurney, M. W. & Marshall, K. C. (1995) *Brain Res. Dev. Brain Res.* **84,** 130–141.
32. Dinsmore, J., Ratliff, J., Deacon, T., Pakzaban, P., Jacoby, D., Galpern, W. & Isacson, O. (1996) *Cell Transplant.* **5,** 131–143.
33. Damjanov, I. (1993) *Int. J. Dev. Biol.* **37,** 39–46.
34. Brinster, R. L. (1993) *Int. J. Dev. Biol.* **37,** 89–99.
35. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. S. (1997) *Nature (London)* **385,** 810–813.
36. Shihabuddin, L. S., Hertz, J. A., Holets, V. R. & Whittemore, S. R. (1995) *J. Neurosci.* **15,** 6666–6678.
37. Sheen, V. L. & Macklis, J. D. (1995) *J. Neurosci.* **15,** 8378–8392.
38. Suhonen, J. O., Peterson, D. A., Ray, J. & Gage, F. H. (1996) *Nature (London)* **383,** 624–627.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 48/00, C12N 5/00, C12Q 1/02, 1/68 | A1

(11) International Publication Number: WO 97/02049

(43) International Publication Date: 23 January 1997 (23.01.97)

(21) International Application Number: PCT/US96/11304

(22) International Filing Date: 5 July 1996 (05.07.96)

(30) Priority Data:
08/499,093 6 July 1995 (06.07.95) US

(71) Applicant: EMORY UNIVERSITY [US/US]; 2009 Ridgewood Drive, Atlanta, GA 30322 (US).

(72) Inventor: LUSKIN, Marla, B.; 1469 Hampton Glen Court, Decatur, GA 30033 (US).

(74) Agents: SELBY, Elizabeth et al.; Needle & Rosenberg, Suite 1200, 127 Peachtree Street, N.E., Atlanta, GA 30303 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: NEURONAL PROGENITOR CELLS AND USES THEREOF

(57) Abstract

The present invention provides an isolated cellular composition comprising greater than about 90 % mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells. Also provided are methods of treating neuronal disorders utilizing this cellular composition.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

NEURONAL PROGENITOR CELLS AND USES THEREOF

This invention was made with government support under NIH grant number NS 28380 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to an isolated cellular composition comprising a substantially homogeneous population of mammalian neuronal progenitor cells. Additionally, the present invention relates to methods of delivering biologically active molecules to a mammalian brain by transplanting the cellular composition to the brain.

Background Art

Because mammalian neurons are generally incapable of dividing when mature, sources of dividing neuronal cells have been sought. Several difficulties have arisen, however, in identifying sources of dividing cells that generate neurons because neuronal progenitor cells frequently fail to express neuronal markers and because heterogeneous populations of cells (including neuronal and non-neuronal cells) generally arise and often individual progenitor cells can give rise to neurons and non-neuronal cells.

Neoplastic cell lines and immortalized neural precursors have been used to provide relatively homogeneous populations of cells. Because these cells are rapidly dividing, they generally show a limited ability to fully differentiate into cells with a neuronal phenotype. For example, PC12 cells derived from a pheochromocytoma fail to differentiate or maintain a differentiated state in culture in the absence of nerve growth factor (NGF). (Green and Tischler, *Advances in Cellular Neurobiology,* S. Federoff and L. Hertz, eds. (Academic Press, N.Y.), (1982). Additionally, these cells are tumor-derived and have neoplastic characteristics. Furthermore, a number of immortalized neural precursor cell lines generate a heterogeneous population of cells.

Similarly, embryonal carcinoma cell lines have been differentiated in culture under special conditions. NT2 cells, derived from a teratocarcinoma, will give rise to

cells that differentiate in culture only following extended treatment of the parent cells with retinoic acid. The NT2 cells, however, differentiate into both neuronal and non-neuronal cell types. The resulting mixed culture must be treated with mitotic inhibitors and then the cells replated to remove the dividing non-neuronal cells and approach a relatively pure population of neuronal cells. (U.S. Patent No. 5,175,103). These relatively pure neuronal cells nonetheless are tumor-derived and have neoplastic characteristics.

Sources of neuronal precursors from adult and neonatal mammalian nervous systems have generally resulted in similar problems with heterogeneity. Reynolds and Weiss, *Science* 255:1707 (1992), have cultured cells from the adult striatum, presumably including portions of the subventricular zone. The cells were cultured in the presence of epidermal growth factor (EGF) and allowed to form large cell clusters, which were termed "neurospheres." The spheres were then dissociated and the cells were cultured in the presence of EGF. The resulting cell cultures consisted of a mixture of post-mitotic neurons, glia, and subependymal cells. Thus, by these means, some of the newly-generated cells were induced to differentiate into neurons; however, the proportion of neurons obtained is low by this method. Others have been able to induce some neuronal proliferation from cultures of the neonatal telencephalon, by administration of fibroblast growth factor. Like the method of Reynolds and Weiss, this neonatal source also results in low proportions of neurons compared to non-neuronal cells. Relatively pure populations of neuronal cells can be achieved by these methods only following treatment with mitotic inhibitors. Therefore, the relatively pure neuronal cells are post-mitotic.

The subventricular zone is known to be a source of certain dividing cells in the nervous system. However, the subventricular zone has been viewed predominantly as a source of glia and not neurons (Paterson *et al., J. Comp. Neurol.*, 149:83, 1973; LeVine and Goldman, *J. Neurosci,* 8:3992, 1988; Levison and Goldman, *Neuron* 10:201 (1993). This was the consensus concerning the intact, *in vivo* subventricular zone. Luskin, however, *(Neuron,* 11:173 (1993)) found that a discrete region of the intact subventricular zone produced numerous neurons that differentiated into olfactory bulb neurons *in vivo*. Nevertheless, other investigators who have cultured cells derived from

the neonatal subventricular zone have shown that the vast majority of these cells become glia when cultured (Vaysse and Goldman, *Neuron*, 4:833, 1990; Lubetzki *et al.*, *Glia*, 6:289, 1992). Lois and Alvarez-Buylla, *Proc. Natl. Acad. Sci.*, 90:2074, (1993) cultured explants of the subventricular zone from adult mammalian forebrain, and found numerous neurons but still a preponderance of glia.

Thus, a simple means of obtaining a composition of cells having a high percentage of neuronal progenitor cells and a correspondingly low percentage of non-neuronal cells is needed. Such a composition and method for achieving the composition would offer several advantages over prior compositions and methods. For example, the time required to obtain a purified population of neurons would be reduced. Dividing cells can be manipulated through gene transfer. In addition, neuronal cells which differentiate and eventually cease dividing result in a decreased likelihood of tumor formation when transplanted into a host nervous system. Glia, in contrast to neurons, can be highly proliferative when given certain signals and can even form gliomas. Neoplastic cell lines can similarly result in tumor formation.

In contrast to the above-described studies which support that only glia arose from the cultured telencephalic subventricular zone or that only a low fraction of neurons could be obtained under particularly favorable conditions, the present invention provides an isolated cellular composition comprised of a substantially homogeneous population of mammalian, non tumor-derived neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells. The ability of these cells to divide is atypical because, with few exceptions, most cells expressing neuron-specific cell markers are post-mitotic cells. Also, the present composition comprises an isolated population of cells of such homogeneity that greater than about 90%, and preferably greater than about 95%, of the neuronal progenitor cells express a neuron-specific marker and can give rise to progeny which can differentiate into neuronal cells.

SUMMARY OF THE INVENTION

The present invention provides an isolated cellular composition comprising greater than about 90%, and preferably greater than about 95%, mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells.

The instant invention additionally provides a method of delivering a biologically active molecule produced by the neuronal progenitor cells, or their progeny, or mixtures thereof, of a cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells to a region of a mammalian brain, comprising transplanting the cellular composition into the region of the brain, thereby delivering a biologically active molecule produced by the cells or their progeny to the region.

Additionally, the present invention provides a method of delivering a biologically active molecule produced by the neuronal progenitor cells, or their progeny, or mixtures thereof, of a cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells and which are transfected with an exogenous nucleic acid that functionally encodes a biologically active molecule to a region of a mammalian brain comprising transplanting the cellular composition into the region of the brain, thereby delivering the biologically active molecule produced by the cells or their progeny to the region.

The present invention further provides a method of treating a neuronal disorder characterized by a reduction of catecholamines in the brain of a mammal, comprising transplanting into the brain a cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells, or their progeny, or mixtures thereof, thereby providing a source of catecholamines to the brain and treating the disorder.

Also provided by the present invention is a method of treating Alzheimer's disease in a subject comprising transplanting into the brain of the subject a cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells and which are transfected with an exogenous nucleic acid that functionally encodes a biologically active molecule that stimulates cell division or differentiation, that promotes neuronal survival, or that functions in the synthesis of a neurotransmitter, or their progeny, or mixtures thereof, thereby treating Alzheimer's disease.

The present invention additionally provides a method of treating a neuronal disorder characterized by a reduction of γ-aminobutyric acid in the brain in a mammal, comprising transplanting into the brain a cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells, or their progeny, or mixtures thereof, thereby providing a source of γ-aminobutyric acid to the brain and treating the disorder.

Also provided by the present invention is a method of screening for a marker of neuronal cells comprising obtaining the neuronal progenitor cells of a cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells, and detecting the presence of a marker in the neuronal progenitor cells that is not present in non-neuronal cells, the marker present in the neuronal progenitor cells that is not present in the non-neuronal cells being a marker of neuronal cells.

The present invention also provides a method of detecting a neuronally expressed gene comprising obtaining a cDNA library from the neuronal progenitor cells of a cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells, obtaining a cDNA library from a non-neuronal cell, determining the presence at higher levels of a cDNA in the library from the neuronal progenitor cells than in the non-neuronal cell, the presence at

higher levels of a cDNA in the library from the neuronal progenitor cells indicating a neuronally expressed gene.

The present invention further provides a method of obtaining an isolated cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuronal marker and which can give rise to progeny which can differentiate into neuronal cells, comprising isolating cells from the portion of a mammalian brain that is the equivalent of the anterior portion of the subventricular zone at the dorsolateral portion of the anterior-most extent of the region surrounding the ventricle of a neonatal rat brain and culturing the isolated cells in the absence of mitotic inhibitors.

The instant invention also provides an isolated cellular composition comprising greater than about 50% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which give rise to progeny which can differentiate into neuronal cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the homotopic transplantation procedure. (A) shows the SVZa, situated between the antero-lateral portion of the lateral ventricle and the overlying corpus callosum, microdissected from a sagittally sectioned neonatal (P0 - P2) forebrain. (B) shows pieces of tissue containing the neuronal progenitor cells of the SVZa which were collected together, trypsinized, washed and mechanically dissociated by trituration into single cells or small clumps. (C) shows the cell suspension which was carefully washed, evaluated for viability, then labeled by the fluorescent, lipophilic dye PKH26 or BrdU to ensure the unequivocal identification of transplanted SVZa cells in the host brain. (D) shows the dissociated, PKH26-labeled SVZa cells stereotaxically placed into the SVZa of a host brain.

Figure 2 shows the heterotopic transplantation procedure for transplanting P0-P2 SVZa neuronal progenitor cells into the neonatal striatum. (A) shows a representative drawing of a parasagittal section of the neonatal rat forebrain showing the location of the SVZa (black area). The SVZa was microdissected from the P0-P2 rat forebrain using a microknife. (B) shows the individual tissue pieces collected in an

Eppendorf tube and dissociated using fire polished Pasteur pipettes to obtain a single cell suspension of SVZa cells. (C) shows the SVZa cell suspension labeled with PKH26, a lipophilic red fluorescent dye. (To label the SVZa cells with the cell proliferation marker, BrdU, P0-P2 pups were injected intraperitoneally with BrdU. A day later the SVZa was dissected and dissociated into a cell suspension). (D) shows the labeled SVZa cell suspension stereotaxically implanted into the striatum (ST) at P0-P2. CC, corpus callosum; CTX, cerebral cortex; D, dorsal; LV, lateral ventricle; OB, olfactory bulb; P, posterior. Scale bar in (A) = 2 mm and also applies to (D).

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples included therein.

The present invention provides an isolated cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells. Preferably at least about 95%, and more preferably greater than about 98%, of the composition is mammalian, non-tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells. By "isolated," as used in the claims, is meant removed from the mammalian brain. As described herein, a region of the anterior subventricular zone (SVZa) isolated from a mammalian brain is shown herein to provide a cellular composition of greater than about 90% neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells. Compositions can also be obtained having, for example, about 50, 60, 70, 80 or 85% neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells. Preferably, greater than about 95%, or even more preferably, greater than about 98%, of the cells in the composition are neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal

cells. Particularly at the time of isolation, about 98 to 100% of the cells in the composition can be neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells. Thus, the invention provides a substantially homogeneous composition of neuronal progenitor cells.

As used herein, "neuronal cells" or "neurons" includes cells which are post-mitotic and which express one or more neuron-specific markers. Examples of such markers can include but are not limited to neurofilament, microtubule-associated protein-2, and tau, and preferably neuron-specific Class III β-tubulin and neu N. As used herein "neuronal progenitor cells" are cells which can give rise to progeny which can differentiate into neuronal cells, but, unlike neuronal cells, are capable of cell division *in vivo* or *in vitro*, and which also, like post-mitotic neurons, express a neuron-specific marker.

In these compositions, preferably only about 10%, or more preferably about 5%, or even more preferably about 2%, or fewer of the cells in the composition are non-neuronal cells. Non-neuronal cells include cells which express a glia-specific marker, such as glial fibrillary acidic protein (GFAP), or which do not express any neuron-specific markers. Non-neuronal cells can include but are not limited to glial cells, subependymal cells, microglia and fibroblasts and do not include neuronal progenitor cells.

As used herein, the "progeny" of a cell can include any subsequent generation of the cell. Thus, the progeny of a neuronal progenitor cell can include, for example, a later generation neuronal progenitor cell, a later generation cell that has undergone differentiation, or a fully differentiated, post-mitotic neuronal cell.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

The present invention provides a cellular composition comprising mammalian, non-tumor derived cells which express a neuron-specific marker and which can divide. The cellular composition can be isolated from the region corresponding to the anterior portion of the subventricular zone (termed "SVZa" interchangeably herein) region of rat brain as described further herein and exemplified in the Examples below. The

substantially homogeneous composition can be obtained in the absence of treatment with mitotic inhibitors. In addition, the ability of the cells to divide can be achieved in the absence of immortalization techniques. The neuronal progenitor cells can, without being first immortalized, divide for at least two generations. At least about two, preferably at least about five, and more preferably at least about ten or more generations of dividing neurons can result when the isolated cells are placed in standard culture conditions as exemplified in the Examples below.

Additionally, the cells of the substantially homogeneous composition of neuronal progenitor cells can give rise to progeny which can differentiate into neuronal cells. By use of this composition, therefore, one can obtain, in the absence of mitotic inhibitors, a composition comprising greater than 90%, and preferably greater than 95%, and more preferably greater than 98%, of any of the following cells: neuronal progenitor cells, progeny of neuronal progenitor cells and neuronal cells.

The cells comprising the herein described composition can be isolated from the SVZa of the brain of any mammal of interest. For example, cells can be obtained from mouse, rat, pig, monkey and human. Preferred sources can be postnatal rat, pig and mouse and prenatal monkey and human brain, though many other sources will be apparent to the practitioner. The SVZa in rat is the dorsolateral portion of the anterior-most extent of the subventricular zone surrounding the ventricles. It is anterior and dorsal to the striatum. It has a different appearance and whiter coloration than the surrounding structures. In addition, it is more opaque than the overlying corpus callosum, presumably because of the density of cells in the region. In other mammals such as human, monkey and mouse, the corresponding region can be located by both this location within the brain and by these physical characteristics.

The present invention provides a cellular composition wherein at least a portion of the cells are transfected by a selected nucleic acid. The cells can be transfected with an exogenous nucleic acid as exemplified in the Examples below. "Exogenous" can include any nucleic acid not originally found in the cell, including a modified nucleic acid originally endogenous to the cell prior to modification. By "transfected" is meant to include any means by which the nucleic acid can be transferred, such as by infection, transformation, transfection, electroporation, microinjection, calcium chloride

precipitation or liposome-mediated transfer. These transfer methods are, in general, standard in the art (*see, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989)). Preferably at least about 3%, more preferably about 10%, more preferably about 20%, more preferably about 30%, more preferably about 50%, and even more preferably about 75% of the cells, at least initially after the transfection procedure is performed, are successfully transfected. To increase the percentage of transfected cells, multiple transfections can be performed. For example, one can infect cells with a vector of choice, remove the media after infection, reinfect, etc. and repeat the process to achieve the desired percentage of infected cells. Some viruses, for example, can be viable for about two hours at a 37°C incubation temperature; therefore, the infection can preferably be repeated every couple of hours to achieve higher percentages of transfected cells. Other methods of increasing transfected cell number, such as transient transfection (Pear, W.S. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:8392-8396 (1993)), are known and standard in the art.

Any selected nucleic acid can be transferred into the cells. For example, a nucleic acid that functionally encodes a biologically active molecule can be transfected into the cells. Preferable nucleic acids can include, for example, nucleic acids that encode a biologically active molecule that stimulates cell division or differentiation or that promotes neuronal survival such as, for example, growth factors, *e.g.*, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4/5, ciliary neurotrophic factor (CNTF), and factors that block growth inhibitors. Additionally, preferable nucleic acids can include nucleic acids that encode a biologically active molecule that functions in the synthesis of a neurotransmitter, such as tyrosine hydroxylase (TH) and glutamic acid decarboxylase (GAD). The nucleic acid can be in any vector of choice, such as a plasmid or a viral vector, and the method of transfer into the cell can be chosen accordingly. As known in the art, nucleic acids can be modified for particular expression, such as by using a particular cell- or tissue-specific promoter, by using a promoter that can be readily induced, or by selecting a particularly strong promoter, if desired.

The present invention also provides methods for isolating the cellular compositions. Thus, methods are provided for isolating a substantially homogeneous composition in the absence of special culture conditions or treatment with mitotic inhibitors and for transfecting at least a portion of the neuronal progenitor cells or their progeny with exogenous DNA . Specifically, the present invention provides a method of obtaining an isolated cellular composition wherein greater than about 90%, and preferably greater than about 95%, and even more preferably greater than about 98%, of the cells of the composition are non-tumor-derived, neuronal progenitor cells which express a neuronal marker and which can give rise to progeny which can differentiate into neuronal cells, comprising isolating cells from the anterior portion of the subventricular zone (SVZa) of a mammalian brain and culturing the cells in the absence of mitotic inhibitors. As discussed above, sources of such cells can preferably be postnatal rat, pig or mouse and prenatal monkey or human brain. The cells are isolated from the SVZa of the selected mammal, as described herein and exemplified in the Examples. The SVZa is located by both its location, as described and exemplified herein, and its physical characteristics, as described and exemplified herein. The cells can then be cultured in the absence of mitotic inhibitors. Thus, the cellular composition, as isolated, can be substantially devoid (*i.e.*, comprises less than 10%, preferably less than 5%, more preferably less than 2%) of glial and other non-neuronal cells, and thus culture conditions designed to eliminate non-neuronal cells from the compositions can often be omitted. Therefore, the cultured cells are not subjected, for example, to mitotic inhibitors. However, if desired, mitotic inhibitors an be utilized. Additionally, the isolated cells can be transfected with an exogenous nucleic acid so that at least a portion of the population is transfected. Furthermore, the cells of the isolated cellular composition can be immortalized by standard methods, such as transformation, to create a cell line (*see, e.g.*, Gage, F.H. *et al.*, *Annu. Rev. Neurosci.* 18:159 (1995)).

The present invention also provides methods for delivering biologically active molecules produced by the neuronal progenitor cells of the composition or their progeny into a region of the brain by transplantation of the cellular composition. Specifically, the present invention provides a method of delivering a biologically active molecule produced by the neuronal progenitor cells of the composition or their progeny or

mixtures thereof described above (which composition comprises an isolated cellular composition of mammalian, non-tumor-derived, neuronal progenitor cells of which greater than about 90%, preferably greater than about 95%, and preferably greater than about 98%, express a neuron-specific marker and can give rise to progeny which can differentiate into neuronal cells) to a region of a mammalian brain comprising transplanting the cellular composition into the region of the brain, thereby delivering a biologically active molecule produced in the cells to the region. The neuronal progenitor cells of the composition or their progeny or mixtures thereof can be transplanted to a host brain, either without being previously cultured or following culture. Culturing can preferably be performed according to standard conditions for neuronal cells or in defined medium with growth factors, as exemplified herein and known in the art. Cells can be cultured for any desirable length of time. For example, cells can be cultured for several days, which can expand the number of cells. For example, the neuronal progenitor cells can be allowed to divide at least once, more preferably twice, five times or ten times or more prior to transplant. Additionally, the cells transplanted prior to differentiation can divide *in vivo* after transplantation. Furthermore, cells for transplantation can be transfected with an exogenous nucleic acid, and the cells can undergo several rounds of transfection with an exogenous nucleic acid prior to transplantation.

Transplantation can be performed for the purpose of delivering to the host brain biologically active molecules normally produced by the transplanted cells (*i.e.*, endogenously-encoded products) or for the purpose of delivering to the host brain biologically active molecules resulting from exogenously introduced DNA in transfected cells that are then transplanted. The term "biologically active molecules," as described also above, includes but is not limited to synthetic enzymes, neurotransmitters, putative neurotransmitters, neurotrophic factors, and factors that can block inhibitors of cell division and/or differentiation.

Transplanting, as known in the art, can be, for example, a stereotaxic injection of a cell suspension, and this injection can be into either a homotopic or heterotopic brain region. Transplantation can be performed as exemplified in the Examples herein. (Dunnett, S.B. and Björklund, A., eds., *Transplantation: Neural Transplantation-A*

Practical Approach, Oxford University Press, Oxford (1992)) Cells, for example, can be suspended in a buffer solution, or alternatively whole tissue comprising the cellular composition, can be transplanted. Dissociated cell suspensions can maximize cell dispersion and vascularization of the graft. Poor vascularization is a significant factor in poor graft survival. Cells can be labeled prior to transplant, if desired. Multiple transplants can be performed, depending upon the number of transplanted cells desired to be transplanted and the area of the target region that receives the transplanted cells. Transplanted cells can preferably divide *in vivo* after transplantation for a limited number of generations, to create a larger region of neuronal progenitor cells and larger numbers of the cells without generating tumor formation. Additionally, transplanted cells can preferably migrate or disperse somewhat within the brain and thus create a larger region receiving these cells. Furthermore, transplanted cells can preferably eventually differentiate into mature neurons.

The present invention provides a method of treating a variety of neuronal disorders or diseases which the provision of a biologically active molecule can treat. By "treating" is meant causing an improvement in any manifestation of the specific disorder or disease. The disorders include but are not limited to disorders characterized by a reduction of catecholamines (such as Parkinson's Disease), by a reduction of GABA (such as certain forms of epilepsy and Huntington's Disease), or by neurodegenerative conditions (such as Alzheimer's Disease). To treat the specific disorder/disease, transfected or non-transfected cells of the compositions or their progeny or mixtures thereof can be transplanted into the host brain wherein the host brain demonstrates the neuronal disorder. The transplantation provides to the brain biologically active molecules produced by the transplanted cells, whether the molecules are endogenous to the transplanted neuronal progenitor cells or their progeny or whether a nucleic acid encoding the molecules were transfected into the transplanted neuronal progenitor cells or their progeny prior to transplantation. Additionally, for example, the cells can be treated prior to transplantation in a manner to cause increased production of the biologically active molecule. Alternatively the cells can be used as a source of the appropriate growth factors to treat the disease. Relatedly, the cells can be used to

screen for novel growth factors which in turn could be screened for therapeutic potential.

Therefore, in one embodiment, cells can be selected for transplantation that will provide a specific biologically active molecule that will treat the specific disease of the subject. For example, for a subject having a disorder characterized by a reduction of catecholamines (such as Parkinson's Disease (PD)), the substantially homogeneous composition comprising isolated neuronal progenitor cells or their progeny, or mixtures thereof, as described above, can be transplanted, for example, for PD, into the region of the striatum. The transplanted cells need not have an exogenous nucleic acid transfected into them, as at least a portion of the cells can produce catecholamines, particularly dopamine. However, if desired, the cells can be transfected with an exogenous nucleic acid prior to transplantation. For example, recombinant nucleic acids encoding enzymes that produce higher than normal levels of the desired biologically active molecule can be utilized, if desired. Other desirable manipulation of the cells will be apparent to the practitioner, in light of the teachings herein.

Another example is treatment of a subject having a disorder characterized by a reduction of GABA, such as certain forms of epilepsy (*Merritt's Textbook of Neurology*, 9th ed. (L.P. Rowland, ed. Williams and Wilkins, Baltimore, 1995)), and Huntington's Disease (HD) (Martin, J.B. & Gusella, J.F. *Huntington's Disease: Pathogenesis and Management*, New Eng. J. Med. 315:1267-1276 (1986)). These subjects can be treated by transplanting into the brain (*e.g.*, into regions such as the cerebral cortex and striatum) cells of the composition or their progeny or mixture thereof as described herein. These cells need not have an exogenous nucleic acid transfected into them, since a substantial portion of the cells can produce GABA. However, if desired, the cells can be transfected with an exogenous nucleic acid. For example, recombinant nucleic acids encoding enzymes that produce higher than normal levels of the product can be utilized, if desired. Other desirable manipulation of the cells will be apparent to the practitioner, in light of the teachings herein. The cells can be transplanted, for example, into regions such as the hippocampus and/or the cerebral cortex, for epilepsy, and the striatum, for Huntington's Disease.

Another example for treatment is neurodegenerative conditions, for example, Alzheimer's Disease. (R.D. Terry, R. Katzman and K.L. Bick, *Alzheimer's Disease*,

Raven Press, NY (1994)). A cellular composition as described herein comprising cells into which has been transfected, for example, a nucleic acid encoding a biologically active molecule that stimulates cell division or differentiation or promotes neuronal survival (such as growth factors *e.g.*, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4/5 and CNTF, or factors that block growth inhibitors), so as to decrease the amount of degeneration, can be transplanted into the brain of the subject (*e.g.*, into regions such as basal forebrain, hippocampus, and/or cerebral cortex). Other desirable manipulation of the cells will be apparent to the practitioner, in light of the teachings herein. The cells can also be used in conjunction with various growth factors for optimal therapeutic effect. Relatedly the cells can be administered with various growth factors to screen factors for therapeutic value in animal models.

The present invention also provides a method of screening for markers of neuronal cells. Specifically, the present invention provides a method of screening for a marker of neuronal cells comprising obtaining the cellular composition described herein (which composition comprises greater than about 90% or 95% or even 99% neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells), obtaining non-neuronal cells or information concerning the markers of those cells, and detecting the presence of a marker in the cellular composition that is not present in non-neuronal cells, the marker present in the cellular composition that is not present in the non-neuronal cells being a marker of neuronal cells. Thus, markers of the cellular composition can be compared to markers of non-neuronal cells to identify markers present in neurons, exclusively or in greater proportions. The neuron-specific markers can be useful in diagnostic and therapeutic techniques for neuronal diseases.

Additionally, the present invention provides a method of detecting a neuronally expressed gene comprising obtaining a cDNA library from the herein described cellular composition (which composition comprises greater than about 90%, preferably greater than about 95%, and more preferably greater than about 98%, mammalian, non-tumor-derived neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells), obtaining a cDNA

library from a non-neuronal cell, determining the presence at higher levels of a cDNA in the library from the cellular composition than in the non-neuronal cell, the presence at higher levels of a cDNA in the library from the cellular composition indicating a neuronally expressed gene. Thus, cDNA libraries derived from the neuronal composition can be compared to a cDNA library from non-neuronal cells to identify genes expressed exclusively or in greater proportions in neuronal cells. Methods of performing such comparative screenings are known in the art, and thus can be readily performed by the artisan given the teachings herein. The neuron-specific markers could be useful in diagnostic and therapeutic techniques for neuronal diseases.

Utility of the Invention

Because mammalian neurons are generally incapable of dividing when mature, sources of dividing neuronal cells have been sought. The present invention provides a source of such dividing cells. These cells additionally demonstrate characteristics of neuronal cells. Therefore, the cellular composition provides a useful composition for, for example, transplanting healthy cells having a neuronal phenotype into subjects whose neurons are degenerating or are not producing normal cellular molecules. The transplanted cells can then provide the deficient molecule(s) to the brain. For example, the present composition can be particularly useful for treating Parkinson's disease (PD), which is characterized by a reduction in catecholamines, by transplanting the inventive cellular composition into the brains of subjects having PD. The transplanted cells can then provide catecholamines to the brain. Another example in which the present composition can be useful is in treating Huntington's Disease or in forms of epilepsy characterized by a reduction in GABA, because these cells can provide GABA to a brain into which they are transplanted. Furthermore, the composition can be useful in providing the desired product of any nucleic acid into the central nervous system. Any desired nucleic acid can be transfected into the neuronal progenitor cells of the composition and transplanted into the central nervous system. An example of a disease that can be treated by such a method is Alzheimer's disease (AD). Cells having a nucleic acid encoding, for example, a growth factor or a neurotrophic factor, can be injected into the brains of AD patients to decrease or prevent degeneration in the brain.

The present compositions additionally can be used to screen for markers of neuronal cells and can be used to further characterize and identify new neuronal cells. The markers can be used for example to detect or treat disease conditions or to identify the anterior portion of the subventricular zone in mammals. Such cells can also be utilized to screen for compounds that affect neuronal cells, either positively or adversely. In this manner, compounds (e.g. novel growth factors) for treating neuronal disorders can be screened, and compounds harmful to neurons can be determined. Many other uses in diagnosis and treatment of neuronal diseases will be apparent to the artisan. The invention can be utilized in therapeutic treatment of any neuronal disease or disorder in which the provision of a healthy neuron and/or a neuron expressing a desirable gene can alleviate some effects of the disease or disorder. Thus, it can have widespread uses, as will be apparent to the skilled artisan given the teachings herein.

The cells can also be used to produce neuronal growth factors for therapy or use as research tools in cell differentiation. The cells themselves can also be used as a research tool to study cell growth and differentiation.

The present invention is more particularly described in the following Examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

Example 1

Microdissection and dissociation of SVZa cells: A method was devised to microdissect the SVZa from parasagittal sections of the newborn rat brain. To harvest SVZa cells, P0-P1 Sprague-Dawley pups were anesthetized on ice, decapitated and their heads placed in cold sterile Ham's F-10 medium (Sigma). After removing the skull, the brain was placed in fresh medium and bisected at the midline. Under the dissecting microscope approximately 2 mm thick parasagittal sections were taken from the midline of the hemispheres and the SVZa microdissected as illustrated in Figure 1. The SVZa is the dorsolateral portion of the anterior-most extent of the region surrounding the ventricles. It is anterior and dorsal to the striatum. The SVZa can be distinguished from

the surrounding structures by its position relative to the ventricle as well as by its coloration and texture. SVZa is white and more opaque than the overlying corpus callosum because it is so cell dense relative to the corpus callosum. The SVZa also appears more dense and uneven because of the cell density. In the neonatal rat, the SVZa can be found at approximately 2.0 mm anterior to bregma, 1.0 mm lateral to the midline and 2.0 mm deep to the pial surface.

Pieces of SVZa tissue from several (7-15) pups were pooled in a sterile test tube containing approximately 5 ml of Hank's balanced salt solution (HBSS). The pieces were incubated for 20 min at 37° C in a 0.1% trypsin and 0.01% DNase in HBSS and washed with medium containing 0.04% DNase in HBSS. The last wash volume was brought up to 5 µl per dissected tissue piece, resulting in 10^5-10^6 cells/ml. To achieve relatively even dissociation into single cells and small clumps, the tissue was thoroughly triturated.

Before transplantation or culture, cell viability was determined using the fluorescent FDA/PI (fluorescein diacetate/propidium iodide) method providing positive identification of living (green) and dead (red) cells. A viability of 80-95% has been routinely obtained from the freshly prepared cell suspensions.

Example 2

Cell labelling *in vitro*: In order to visualize cells transplanted into a host brain, the cells can be labelled with the lipophilic membrane bound dye, PKH26, which fluoresces red with a 551 nm excitation and 567 nm emission, can be used to label the dissociated SVZa cells immediately prior to transplantation. For the SVZa cells, the freshly dissociated cell suspension was labelled with PKH26 (4 µM dye in diluent C, Sigma) for 3-5 min. Virtually all cells become intensely labeled.

In some experiments, BrdU (5 mg BrdU/ml of 0.007 N NaOH in 0.9% NaCl), a cell proliferation marker, has been used to label dissociated SVZa cells prior to transplantation. Using this labelling method, dividing cells can be visualized after transplantation according to the procedure described by Menezes and Luskin, *J. Neurosci.* 14:5399 (1994). Specifically, bromo-deoxyuridine (BrdU) was added to the

culture media, and then 1 to 24 hours later the cultures were fixed as described above and stained with antibodies to BrdU to reveal the presence of labeled cells. After fixation, the cultures were washed with 0.01 M PBS and treated with 2N HCl at 60°C to fragment the DNA followed by acid neutralization in 0.01 M borate buffer, pH 8.3. After a thorough wash with PBS and application of blocking serum (10% normal goat serum with 0.01% Triton X-100 in 0.01 M PBS), the cultures were incubated overnight with a monoclonal antibody to BrdU (α-BrdU, Accurate, NY), at 4°C using a 1:500 dilution. Afterwards the cultures were rinsed with 0.1 M PBS and incubated with a rhodamine conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch, PA) at a 1:200 dilution for 1 hour at room temperature, washed in 0.1 M PBS and coverslipped using Vectashield (Vector, CA). BrdU-positive cells display a red fluorescent nucleus.

Example 3

Cell culture: The isolated SVZa cells in culture are essentially all neuronal, i.e., they are immunoreactive when stained with neuron-specific markers. To ascertain the phenotype of the harvested and dissociated SVZa cells, they were plated on uncoated glass microscope slides or poly-D-lysine or polyornithine coated glass slides and cultured in either full strength Ham's F10 medium (Sigma) or Dulbecco's minimal essential medium DMEM (Sigma) supplemented with 10% fetal calf serum or 1:1 ratio of Ham's F10 medium:DMEM, at 37°C in 7% CO_2. Specifically, following dissociation, the cells were centrifuged at 700 rpm for 7 min, the pellet redispersed in new medium and the number of cells estimated using a hemacytometer. Approximately 3.32×10^3 cells were added to each well of the glass chamber slides (LabTek 16 well). Alternatively, cells were plated at a density of 3.3 or 5.9×10^5 cells/cm^2. Each well was coated with 10 µg/ml of poly-D-lysine (P-7280, Sigma) for 1 h at 37°C in the incubator, rinsed 3 times with distilled water and air dried in the culture hood. Alternatively, the cells were plated on 10 µg/ml of mouse laminin (23017-015, Gibco), on 500 µg/ml poly-L-ornithine (P-3655, Sigma) or on a combination of both.

One to eight days later the SVZa cultures were fixed for 20 min in 4% paraformaldehyde and 0.12 M sucrose in 0.1 M PBS, rinsed in cold PBS, permeabilized

with 100% ethanol, rehydrated in an ethanol series and rinsed in PBS. After incubation in 50 mM glycine and three rinses in cold PBS, blocking serum (0.5% normal goat serum and 0.01% Triton X-100 in 0.1 M PBS) was applied for 1 hour. Cells were incubated overnight with a 1:500 dilution of the mouse monoclonal antibody TuJ1, a neuron-specific antibody recognizing class III β-tubulin (Lee *et al., Proc. Natl. Acad. Sci.* 87:7195 (1990)); supplied by Dr. A. Frankfurter, University of Virginia, Charlottesville, VA) and a rabbit polyclonal antibody (GFAP; Dako) to glial fibrillary acidic protein (Bignami *et al., Brain Res.* 43:429 (1972)) at a dilution of 1:500. Cells were then rinsed in 0.1 M PBS and incubated for an hour in a mixture of secondary antibodies including fluorescein goat anti-mouse (Jackson, 1:100) and rhodamine goal anti-rabbit (Jackson, 1:200), washed in 0.1 M PBS, pH 7.4, coverslipped using Vectashield (Vector, CA) and examined by epifluorescence microscopy.

To ascertain definitively the identity of the microdissected cells prior to transplantation, cells were plated and stained for cell-type specific markers to characterize them. Characterizing the identity of the cells was done to determine the purity of the dissected cells and whether the microdissected cells contained progenitors for glia. As described above, the viability of the dissociated cells prior to plating was quite high; between 80-95 per cent and often higher than 95%. When viewed by bright-field and phase microscopy within the first few hours after plating, the vast majority of cells adhered to the surface of the glass and some even extended one or two processes from their cell bodies. This indicates that some of the cultured cells began to differentiate almost immediately after plating. TuJ1, an antibody that recognizes neuron-specific class III β-tubulin (Lee *et al., Proc. Natl. Acad. Sci.* 87:7195 (1990)), was used to identify cells with a neuronal phenotype and an antibody to GFAP to distinguish astrocytes, a cell type commonly derived from other regions of the neonatal subventricular zone (Privat, *Int. Rev. Cytol.* 40:281 (1975); Levison and Goldman, *Neuron* 10:201 (1993); Luskin and McDermott, *Glia* 11:211 (1994)).

After one day *in vitro* (1 DIV) all or nearly all of the cultured SVZa cells stained with TuJ1. When viewed by bright-field and phase microscopy within the first few hours after plating, the vast majority of cells adhered to the surface of the glass slide and

some even extended one or two processes from their cell bodies. This indicates that some of the plated cells began to differentiate almost immediately after plating.

After 24 hours in culture, the majority of the cultured cells either occurred in small clusters containing 2-4 cells or as individual cells with a bipolar or occasionally multipolar morphology. Interestingly, the overwhelming majority of clustered and individual cells exhibited distinct TuJ1 immunoreactivity, apparent in the somatic cytoplasm and cell processes. At this stage, GFAP-positive cells in the cultures were rarely seen. The result showed that the plated cells possess a pronounced neuronal identity. This result also indicated that only the SVZa was included in the dissection. If this were not the case, GFAP-positive cells would be expected.

Cells were also stained at intermediate times up to 8 days in culture to discern what proportion of the cells exhibit exclusively a neuronal phenotype. At 8 days, the cultured cells occurred in small clumps or were loosely arranged and that the cells now extended numerous intermingling processes. Again, nearly all of the cells expressed prominent TuJ1 immunoreactivity. As in the short-term cultures, glia, as signified by GFAP-immunoreactivity, represented less than 5% of all cultured cells. These findings demonstrated that the region of the SVZa which contains a seemingly pure population of neuronal progenitor cells can be isolated.

Since many types of neurons exhibit substrate-dependent process outgrowth, the ability of SVZa-derived cells to extend processes was tested on different substrates. SVZa cells were found to extend processes on poly-D-lysine at 10 µg/ml and on poly-L-ornithine (or on poly-D-L-ornithine) and exhibited monopolar, bipolar and multipolar morphologies. However, in contrast to cerebellar granule neurons, on 10 µg/ml laminin, SVZa cells did not sprout.

Another unexpected property of the cultured SVZa cells is that they proliferate in culture. This was surprising because most cells expressing neuron-specific cell markers are post-mitotic cells (Moody *et al.*, *J. Comp. Neurol.* 279:567 (1989); Menezes and Luskin, *J. Neurosci.* 14:5399 (1994). Furthermore, it is often difficult to establish conditions under which cells giving rise to neurons can divide in culture, especially when plated at low density, as in the present example. (Reynolds and Weiss, *Science*

255:1707 (1992). Not only did the cultured SVZa cells divide immediately after plating, but they also divided several days after they have been cultured.

To demonstrate that cultured SVZa cells undergo division, the cell proliferation marker bromo-deoxyuridine (BrdU) was added to the culture media, and then 1 to 24 hours later the cultures were fixed as described above and stained with antibodies to BrdU to reveal the presence of labeled cells. After fixation, the cultures were washed with 0.01 M PBS and treated with 2N HCl at 60°C to fragment the DNA followed by acid neutralization in 0.01 M borate buffer, pH 8.3. After a thorough wash with PBS and application of blocking serum (10% normal goat serum with 0.01% Triton X-100 in 0.01 M PBS), the cultures were incubated overnight with a monoclonal antibody to BrdU (α-BrdU, Accurate, NY), at 4°C using a 1:500 dilution. Afterwards the cultures were rinsed with 0.1 M PBS and incubated with a rhodamine conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch, PA) at a 1:200 dilution for 1 hour at room temperature, washed in 0.1 M PBS and coverslipped using Vectashield (Vector, CA). BrdU-positive cells display a red fluorescent nucleus.

Example 4

Homotopic transplantation of SVZa cells: To investigate the migratory behavior of homotopically transplanted SVZa-derived cells, dissociated donor rat SVZa cells were implanted in the neonatal SVZa of a rat host. The purpose of the experiment was to determine if transplanted cells are able to read the same guidance cues and attain the same laminar distribution in the host brain as unmanipulated SVZa-derived cells. Dissociated SVZa cells rather than explants of tissue were transplanted to facilitate the integration of the transplanted cells in the host brain.

In order to analyze the migratory behavior of homotopically transplanted SVZa cells, the distribution of transplanted cells at 3 postimplantation time periods was examined: short survivals (after 1 week or less), intermediate survivals (after 2 to 3 weeks) and long survivals (4 weeks or longer). The experiment was performed to find out if the distribution of the transplanted cells matched that of the unmanipulated cells at the various time points chosen for study. From our *in vivo* studies in which PKH26 was directly injected into the SVZa to label its cells, the time periods chosen for analysis

correspond to when SVZa-derived cells would occur predominantly in the pathway, subependymal zone of the olfactory bulb and overlying granule cell layer, and when they are in their final positions in the granule cell and glomerular layers.

Short-term survival

To compare the overall distribution and dynamics of cell movement by unmanipulated SVZa-derived cells to that of transplanted SVZa cells, dissociated PKH26-labeled SVZa cells were injected into the host SVZa. To visualize PKH26 labelled cells in vivo, animals were perfused with 4 % paraformaldehyde, their brains removed, and sectioned on a Vibratome. Serial 100 μm sections were mounted and examined by fluorescence microscopy for PKH26-labeled cells. The subsequent position and morphology of the cells were examined within one week after transplantation.

Examination of host brains 1 day after transplantation revealed that the injection site was usually centered in the SVZa and that it usually contained a high density of PKH26-labeled cells. At the injection site the red fluorescing PKH26-labeled cells were small and round. These cells usually occurred as individual cells or in small clumps, resembling freshly dissociated cells.

The path of migration demonstrated by transplanted SVZa cells matches precisely the path followed by unmanipulated SVZa-derived cells. It constitutes a long pathway connecting the SVZa to the center of the olfactory bulb measuring several millimeters. At progressively longer times after transplantation the distribution of labeled cells extended further from the site of implantation.

By two days after transplantation, a continuous stream of cells was observed coming from the rostral wall of the anterior horn of the lateral ventricle (SVZa) to the vertical limb of the pathway. By four days after transplantation the labeled cells were in the horizontal arm of the pathway, and some cells reached the central part of the olfactory bulb. At the end of the first week after transplantation, migrating cells were found evenly distributed throughout the subependymal layer extending from the SVZa to the middle of the olfactory bulb. Moreover, as found for the unmanipulated SVZa-derived cells, the transplanted cells were strictly confined to the well-defined pathway

characterized by a region of high cell density. This demonstrates that the transplanted PKH26-labeled SVZa cells faithfully acknowledge the boundaries of the migratory pathway and do not deviate from it.

Fluorescence microscopy revealed that the majority of transplanted PKH26-labeled cells have a round soma, and that some have a relatively short and thick process extending toward the olfactory bulb. Within the subependymal zone of the olfactory bulb, many transplanted cells have an oval or spindle-shaped soma with a clear, unlabeled nucleus. In contrast to the unmanipulated SVZa-derived cells, at this stage only a low number of dye-labeled cells revealed processes. One possibility to account for the differential labeling of SVZa-derived cells is that perhaps the PKH26 does not label the transplanted cells in their entirety. Alternatively, perhaps some transplanted cells lack fully developed processes. In this case the transplanted cells may be able to reach the bulb by becoming incorporated into the stream of unmanipulated SVZa-derived cells which are also traveling to the olfactory bulb.

Intermediate survival

Distribution of transplanted cells in the migratory pathway and granule cell layer of the olfactory bulb. By two weeks after transplantation some of the transplanted cells had advanced into the granule cell layer of the olfactory bulb. It appeared as though the labeled cells had moved from the subependymal layer of the bulb into the overlying granule cell layer. Concomitantly, there was a striking reduction in the proportion of transplanted cells in the more caudal parts (vertical limbs) of the migratory pathway. By three weeks after transplantation a greater proportion the donor cells had entered the granule cell layer, leaving fewer in the subependymal zone and pathway distal to the olfactory bulb.

When the transplanted cells turned radially from the subependymal zone towards the granule cell layer, some of them began to differentiate into granule cells, revealing two PKH26-labeled processes. The transplanted cells within the granule cell layer, which presumably are undergoing differentiation, had the characteristic bipolar morphology of maturing, unmanipulated granule cells. The range of mature and immature morphologies seen among the PKH26-labeled cells 2-3 weeks after homotopic

transplantation indicates that the cells are at various stages of differentiation. In fact, some of the PKH26-labeled cells in the granule cell layer appeared to be still en route to the glomerular layer, judging by their spindle-shaped cell soma which is characteristic of migrating neurons.

In some experiments BrdU incorporation was used to label SVZa cells before transplantation. BrdU-labeled cells were visualized according to the procedure described by Menezes and Luskin *J. Neurosci* 14:5399 (1994). In brief, brains were perfused with 4% paraformaldehyde and then cryoprotected overnight in 20% sucrose in 0.1 M phosphate buffered saline (PBS). The brains were embedded in Tissue Tek O.C.T. Compound, sagittally sectioned on a cryostat at 18 - 20 μm and mounted on slides before processing for the presence of BrdU. The sections were washed with 0.01 M PBS and treated with 2N HCl at 60°C to fragment the DNA followed by acid neutralization in 0.01 M borate buffer, pH 8.3. After a thorough wash with PBS and application of blocking serum (10% normal goat serum with 0.01% Triton X-100 in 0.01 M PBS), the sections were incubated overnight with a monoclonal antibody to BrdU (α-BrdU, Accurate, NY), at 4°C using a 1:500 dilution. Afterwards the sections were rinsed with 0.1 M PBS and incubated with a rhodamine conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch, PA) at a 1:200 dilution for 1 hour at room temperature, washed in 0.1 M PBS and coverslipped using Vectashield (Vector, CA). BrdU-positive cells display a red fluorescent nucleus. The distribution of transplanted BrdU-labeled cells matched the distribution of PKH26-labeled cells when examined after the same survival period. Two weeks after transplantation, fluorescence microscopy revealed the presence of intensely labeled BrdU-positive cells predominantly in the portion of the migratory pathway close to the olfactory bulb (horizontal limb) and in the subependymal zone of the bulb, although a few had advanced into the overlying granule cell layer. Thus, even though the BrdU labeling does not reveal the precise morphology of the transplanted cells, it clearly reveals their position.

Long-term survival

Both PKH26 and BrdU labeling procedures were used to unequivocally identify the transplanted SVZa-derived cells. In particular, there were concerns that over time

the PKH26 dye intensity may diminish. Therefore, most conclusions were based on the analysis of BrdU-labeled cells.

Previous studies showed that four weeks after an injection of retrovirus into the SVZa that the SVZa-derived cells have achieved their final laminar distribution (Luskin, *Neuron* 11:173 (1993)). In these experiments, a similar laminar distribution of transplanted cells was found. When compared with the intermediate survival, significantly higher numbers of transplanted cells were distributed throughout the granule cell layer. Another group of cells, most likely periglomerular cells, were found encircling the glomeruli. A few transplanted cells still occupied the rostral half of the subependymal layer of the olfactory bulb 4 weeks after transplantation. Thus, the sequential changes in the migratory pattern of unmanipulated SVZa cells seems to be matched by the homotopically transplanted cells. This suggests that they are able to discern the same set of guidance cues.

Quantitative analyses showed that the ratio between labeled cells in the glomerular layer and granule cell layer after transplantation was identical to what occurs in the unmanipulated brain (Luskin, *Neuron* 11:173, (1993)). Seventy-five percent of the transplanted cells ended up in the granule cell layer or adjacent to it and the other twenty-five percent were found in the glomerular layer of the olfactory bulb. Collectively, these findings suggest that transplanted SVZa-derived cells are not only able to adopt the same migratory route as their counterparts originating from the host SVZa but that they are also able to acquire the same laminar distribution between the granule cell and glomerular layers in the olfactory bulb.

Example 5

Heterotopic transplantation of SVZa cells into neonatal cerebellum, ventricular zone of embryonic telencephalon, or areas adjacent to the anterior portion of the subventricular zone: To make injections into the external granular layer of the neonatal cerebellum, a small incision through the skull overlying the midbrain and the hindbrain can be made and labeled SVZa cells can be injected using a Hamilton syringe into a position just beneath the meninges (Gao and Hatten, *Science* 260:367 (1993)).

To make injections into the ventricular zone of the embryonic telencephalon the procedure described by Dunnett and Bjorklund in *Transplantation: Neural Transplantation-A Practical Approach,* Oxford Univ. Press, Oxford (1992), can be followed. In brief, under deep anesthesia the abdominal wall of a pregnant dam can be incised. The uterine horns can be exposed and each fetus transilluminated with the fiberoptic tube. A pipette containing labeled SVZa cells can be inserted through the uterine wall, amniotic sac, and the fetal skull into the ventricular zone overlying the cerebral cortex.

To investigate the behavior and distribution of SVZa cells transplanted into areas adjacent to the anterior portion of the subventricular zone, SVZa cells were transplanted into position lying either posterior or lateral to the SVZa of the host. Retrovirus injections had shown that only when the injections were within the SVZa did the labeled cells end up in the olfactory bulb and become neurons (Luskin, *Neuron* 11:173 (1993), Luskin and McDermott, *Glia* 11:211 (1994)). Of the four animals used in this experiment, no labeled cells were found in the migratory pathway or in the olfactory bulb following the nonSVZa injections, confirming that SVZa provides certain positional information to guide SVZa-derived cells to the olfactory bulb.

The phenotypic identity of unmanipulated SVZa-derived cells in the mature (> 6 weeks) olfactory bulb has been analyzed. The phenotype of SVZa-derived cells can be classified according to their morphology (Pinching and Powell, *J. Cell Sci.* 9:305, 347, 379 (1971)) and the neurotransmitter candidates they contain (Bartolomei and Greer, *Neurosci. Abst.* 19:125 (1993). Halasz *et al. Brain Res.* 167:221 (1979) has shown that essentially all granule cells contain GABA, as do many periglomerular cells. Periglomerular cells are also known to express tyrosine hydroxylase, the rate limiting step in the synthesis of dopamine (McLean and Shipley, *J. Neurosci.* 8:3658 (1988). Moreover, Gall *et al. J. Comp. Neurol.* 266:307 (1987), and Kosaka *et al. Brain Res.* 343:166 (1985) have independently shown the colocalization of GABA and TH in subsets of periglomerular cells. Furthermore, since Celio *Neurosci.* 35:375 (1990), Halasz *et al. Neurosci. Letters* 61:103 (1985) and Kosaka *et al. Brain Res.* 411:373 (1987) reported that virtually all periglomerular cells are immunoreactive for calbindin (28K-vitamin-D-dependent calcium binding protein), calbindin immunoreactivity can be

determined in unmanipulated and transplanted BrdU-labeled SVZa cells situated in the glomerular layer express calbindin. Furthermore, the phenotype acquired by heterotopically transplanted SVZa-derived cells in the cerebellum and cerebral cortex, and that acquired by ventricular zone and EGL cells in the olfactory bulb can be examined.

Example 6

Double-labeling: Following transplantation of BrdU-labeled SVZa cells into the SVZa, as described above, procedures have been devised to reveal the presence of BrdU and transmitter candidates or their synthetic enzymes using double label procedures on 20 μm cryostat sections. Following perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) brains were removed, equilibrated in 20 - 30 % sucrose in 0.1 M phosphate buffer overnight and then cut sagittally or coronally at a thickness of 20 μm on a cryostat. Sections were washed in 0.1 M PBS, treated with 2N HCl at 45 - 50°C for 15 minutes and subsequently rinsed with 0.1 M borate buffer, pH 8.3 for 15 minutes. Sections were then incubated in 10% normal goat serum in PBS for 30 minutes and then overnight in a mixture of primary antibodies including anti-BrdU (1:500; Accurate, NY) and an antibody to either GABA (1:500; Sigma), TH (1:1000, Eugene Tech, NJ) or calbindin (Sigma, 1:1000 dilution). The next day the sections were rinsed in 0.1 M PBS and incubated for 2 hours in an appropriate mixture of secondary antibodies that contain goat anti-rat IgG conjugated to rhodamine to visualize BrdU immunoreactive cells and FITC conjugated secondaries to identify one of the neurotransmitter candidates. Lastly the sections were rinsed in 0.1 M PBS and coverslipped.

Sections were examined with fluorescence microscopy to identify labeled SVZa cells, and their neurotransmitter phenotype and laminar position determined. The SVZa-labeled cells were evident by their red fluorescence and the transmitter labeling, when present in the same cells by green fluorescence of both unmanipulated and transplanted cells. The percentage of SVZa-derived GABAergic and TH-immunoreactive cells were determined for unmanipulated cells in each layer of the olfactory bulb.

Previous studies have shown that the SVZa-derived cells are neurons based on their morphological features and laminar distribution. To further characterize the SVZa-derived neurons in the olfactory bulb, cell-type specific markers for transmitter phenotype were used. At P20, when most of the SVZa-derived cells have reached their final destination following an SVZa injection of BrdU at P2, BrdU-labeled cells were localized using immunohistochemistry and their neurotransmitter phenotype was assessed using antibodies against gamma-aminobutyric acid (GABA) and the dopamine synthesizing enzyme tyrosine hydroxylase (TH). Using simultaneous indirect immunofluorescence to detect the presence of single- and double-labeled cells, 10% of the SVZa-derived cells were found to be both BrdU- and TH-positive in the glomerular layer and that approximately 67% and 46% of the SVZa-derived cells in the granule cell layer and glomerular layer were GABAergic (GABA- and BrdU-positive), respectively. When analyzed at P20, 28% and 12 % of the periglomerular cells, that arose from a P2 injection of BrdU were TH- and GABA-positive respectively, were found. Similarly, at P20, 11% of the GABAergic neurons in the granule cell layer were generated on P2. These results indicate that the neonatal SVZa is a source of dopaminergic cells destined for the glomerular layer and also a source of GABAergic cells for the granule cell and glomerular layers.

The transmitter phenotype of unmanipulated SVZa-derived cells in the olfactory bulb can now be compared with the transmitter phenotype expressed by homotopically and heterotopically transplanted cells that reach the olfactory bulb after implantation in the SVZa. This can allow determination of whether transplanted cells acquire the same transmitter identity as unmanipulated SVZa-derived cells, or if transmitter candidates expressed by the heterotopically transplanted cells are more representative of the transmitters they ordinarily express. If the heterotopically transplanted cells reach the periglomerular layer and express TH, then conclusions can be drawn that their identity has been respecified; dopamine is ordinarily expressed only by cells of the substantia nigra and olfactory bulb. The phenotype of unmanipulated cells can be compared to the homotopically and/or heterotopically transplanted cells, *i.e.*, those implanted in the striatum.

Example 7

Heterotopic transplantation of cortical and cerebellar cells into neonatal SVZa: In additional experiments, it was investigated whether newly-generated neurons, which usually migrate along radial glia, could navigate the highly restricted path adhered to by SVZa derived cells that appears not to be guided by radial glia. Cerebellar external granule layer (EGL) cells (postnatal) and ventricular zone (VZ) cells (prenatal) were harvested for transplantation. In brief, EGL cells were removed by suction on the surface of the cerebellum or by microdissection and then trypsin and DNase were used to dissociate the cells as described above. To harvest progenitor cells of the E16 VZ, a modified procedure used by McConnell, *Brain Res. Rev.* 13:1 (1988), was employed. Dissociated cells from the VZ of the embryonic day 15 to 17 rat telencephalon or from the EGL of the postnatal day 5 (P5) or P6 cerebellum, were labeled with either the cell proliferation marker BrdU or the fluorescent lipophilic dye PHK26 and stereotaxically implanted into the SVZa of P0-P2 rats. Results showed that heterotopically engrafted VZ cells remained at the site of infection. In contrast, heterotopically transplanted EGL cells traversed the migratory pathway, although most did not migrate away from the middle of the olfactory bulb (OB).

Example 8

Heterotopic transplantation of SVZa cells into the striatum: To maximize the number of labeled SVZa cells obtained for transplantation, P0-P1 donor pups were given 2-3 intraperitoneal injections (6 hours apart) of a BrdU stock solution (5 mg BrdU/ml of 0.007 N NaOH in 0.9% saline; 0.3 ml/pup/injection). The last injection was given one hour before dissection of the donor tissue.

The SVZa cells were dissected and dissociated as described above and the viability of the cell suspension determined as described above. A viability of about 80-95% was obtained, and the cell concentration ranged from 2.9×10^4 to 5.4×10^6 cells/ml. The dissociated cells were labeled with PKH26 by incubating the freshly prepared cell suspension in a 4.0 μM solution of PKH26 dye and diluent C for 3-5 minutes according to the protocol provided by Sigma.

The dissociated and labeled SVZa cells were transplanted into the striatum of P0-P2 pups that were anesthetized by hypothermia. To reduce movement and maximize the consistency of injection coordinates, the head of the pup was placed on a Sylgard contoured mold. (To determine the coordinates for targeting the P0-P2 striatum, PKH26 was directly injected into the brains of four P0-P1 pups. The range of coordinates were chosen by comparing the results obtained from PKH26 injections as well as from a few initial transplantation experiments using implantation of labeled SVZa cells.) The injections were made between 0.8 - 2.0 mm anterior to bregma (A-P) and 1.2 - 2.3 mm lateral to the sagittal sinus (M-L) and 2.3 - 3.5 mm deep to the pial surface (depth). We demonstrated that injections within the following range of coordinates A-P, 1.0 - 1.5 mm; M-L, 1.8 - 2.3 mm and depth, 2.5 - 3.5 mm, were most likely to target the striatum (Table 1) and were in agreement with those used by Abrous et al. (1).

An incision was made through the skin overlying the sagittal suture to expose the skull. A small hole was made through the skull centered around 1.8-2.3 mm lateral to the sagittal suture and 1.0-1.5 mm anterior to the bregma. A 10 µl Hamilton syringe, containing the SVZa cells, attached to a micromanipulator, was lowered approximately 2.5-3.5 mm from the pial surface and 2-4 µl of the labeled cell suspension was injected into the striatum. Following transplantation, the overlying skin was repositioned and sealed with surgical glue and the pup was placed under a heat lamp for recovery before transferring it back to its home cage. Following transplantation the pups were allowed to survive for various time periods before they were perfused. At the time of perfusion the pups were anesthetized with ether and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brains were removed, blocked in the sagittal plane, and post-fixed in the same fixative for at least 1 h before washing with 0.1 M PBS. The BrdU and PKH26-labeled cells were detected as described above.

Table 1: Coordinates for implantation of SVZa cells and their subsequent distribution

Rat #	Age at implantation	Survival (days)	Injection site (mm) AP	M-L	Depth	Amount injected (µL)	PKH26 or BrdU	Labeled cells: in striatum/along striatal boundary
A. Short-term survival								
1	P1	3	0.9	1.8	3.1	4	BrdU	+/-
2	P1	5	0.8	1.7	2.4	3	PKH26	-/-

3	P1	2	0.7	2.0	3.1	3	BrdU	+/-
4	P1	2	1.1	2.0	3.2	3	BrdU	+/-
5	P1	2	1.0	2.2	3.2	3	BrdU	+/-
6	P1	2	1.0	2.3	3.3	3	BrdU	+/-
7	P1	2	0.8	2.0	3.2	3	BrdU	+/-
8	P1	2	1.0	2.0	3.2	3	BrdU	+/-
B. Long-term survival								
I. SVZa cells restricted to the striatum								
1	P0	13	1.5	2.0	2.9	4	PKH26	
2	P1	26	1.0	2.0	3.2	4	PKH26	
3	P1	26	1.0	2.0	2.9	4	BrdU	
4	P1	26	0.8	1.2	3.3	4	BrdU	
II. SVZa cells restricted to the striatal boundary								
5	P1	13	2.0	1.7	2.3	2	PKH26	
6	P0	20	1.2	2.0	2.5	2	PKH26	
7	P1	28	1.2	1.5	3.3	3	BrdU	
III. SVAz cells within the striatum and along the striatal boundary								
8	P2	13	1.0	2.0	3.3	3	BrdU	
9	P1	18	1.2	2.0	3.3	4	BrdU	
10	P0	26	1.0	2.0	3.0	4	PKH26	
11	P1	26	1.0	2.0	3.0	4	PKH26	
12	P1	41	1.2	2.0	3.4	4	BrdU	
IV. SVZa cells present elsewhere in the brain								
13	P2	13	1.0	2.0	3.2	3	BrdU	
14	P2	13	1.0	2.0	3.2	3	BrdU	
15	P1	19	1.0	1.7	2.5	4	PKH26	
16	P1	20	2.0	1.5	3.1	4	PKH26	
17	P1	21	1.8	1.2	3.2	4	PKH26	

The brains of neonatal rat pups received transplants of labeled SVZa cells into the striatum and the ensuing distribution of the transplanted SVZa cells at the time of perfusion (Survival) was mapped. P0-P2 pups were implanted with PKH26- or BrdU-labeled P0-P2 SVZa cells. All the transplants were placed in the right hemisphere while the head was in the Sylgard mold. The reference points for the injection site coordinates were as follows: measured distance anterior to the bregma for the anteriorposterior (A-P) dimension, distance lateral to the sagittal sinus for the mediallateral (M-L) dimension, and distance below the pial surface for the Depth. The presence or absence of labeled cells in the striatum or along the striatal boundary was scored as (+) or (-), respectively. A total of 25 brains were studied. They were grouped into short-term survival (2-5 days; n =8) and long-term survival (>13 days; n = 17). Of the 17 brains in the long-term survival group, 12 brains were used for detailed analysis. In the remaining five brains the transplant was placed superficial to the striatum and was excluded from further consideration. Note that the distribution of the SVZa cells is not related to (1) the amount of cell suspension injected, (2) age of the host at the time of transplantation, or (3) survival time posttransplantation. Also, the presence of transplanted cells along the lateral cortical stream is not correlated with any particular set of coordinates.

Appearance of cells at injection site

Three days after transplanting SVZa cells into P1 striatum BrdU-labeled SVZa cells were readily identified in the middle of the striatum and in some cases also along the injection tract running through the corpus callosum. The presence of labeled cells along the injection tract is probably due to the backflow of the cell suspension or because of a small amount of leakage of the labeled cells during insertion or withdrawal of the Hamilton syringe. The results show complete and heavy staining of the nuclei of the labeled cells soon after transplantation. Many of the BrdU-labeled cells were aggregated near blood vessels. In addition, at this short survival time cells were usually seen adjacent to each other, although a few cells were more dispersed within the striatum and had evidently undergone migration.

Patterns of migration of donor SVZa cells in the host striatum

The unmanipulated SVZa cells generated between P0-P2 migrate several millimeters to the subependymal layer in the middle of the olfactory bulb. By 4 weeks they attain their final position in the granule cell or glomerular layers. The distribution of the labeled SVZa cells in the host striatum was therefore examined at 2 to 4 weeks (long-term survivals, n=17, Table 1) after transplantation to investigate whether the SVZa cells had dispersed from their site of injection. We restricted our analysis to 12 of the 17 injected brains in which SVZa transplants were located in the striatum. The remaining five brains were excluded from further consideration because we concluded that the SVZa cells were not implanted deep enough in the brain. The transplanted cells in these brains were found in the subventricular zone or corpus callosum dorsal to the ventricle overlying the striatum.

We observed three patterns of distribution of transplanted SVZa cells and thus grouped the results from the 12 brains accordingly: 1) cases in which the labeled cells were confined to the striatum; 2) cases in which the labeled cells were situated along the striatal boundary between the striatum and the corpus callosum; and 3) cases in which the labeled cells were present in both of the above-mentioned locations (Table 1). We observed that the distribution of the SVZa cells was not related to the post-transplantation survival time, nor to the age of the host at the time of transplantation. A

striking finding of this study was that the injection site could not be demarcated 2-4 weeks post-transplantation in any of the cases studied; gliosis was not observed around the transplants. In addition, although SVZa cells were seen along the striatal boundary, they were never seen to cross it and migrate into the surrounding cerebral cortex.

Appearance and distribution of SVZa cells restricted to striatum.

The labeled SVZa cells were identified in the striatum in 4 out of 12 animals (Table 1) analyzed. In each brain the SVZa cells within the striatum occurred as individual cells or in small groups of usually no more than 2-4 cells. Large, closely packed aggregates of cells were never observed 2-4 weeks after transplantation, indicating that the cells had migrated away from each other. The labeled cells were frequently found in close proximity to blood vessels. Although the labeled cells were present through the striatum, in the majority of the brains analyzed the labeled SVZa cells were situated closer to the lateral ventricle than to the lateral edge of the striatum.

Amongst the transplanted cells labeled with PKH26, small clumps of 2-4 cells were seen extending processes into the striatum. The BrdU-labeled SVZa cells located in the striatum 2-4 weeks following transplantation were not heavily stained as cells examined 3 days post transplantation. This suggested that the SVZa cells had undergone cell division after transplantation into the striatum. Our observations indicate that the heterotopically transplanted SVZa cells retained their capacity to concurrently divide and migrate.

Unlike other studies in which cells were transplanted into the striatum, glial cells were rarely seen associated with the transplants. The presence of glial cells, a sign that the host striatum is reacting to the local trauma produced by the implantation procedure, was absent in the SVZa transplants and could be attributed to the younger age of the donor and host animals used. The absence of the glial barrier could be partially responsible for the dispersion of the transplanted SVZa cells within the striatum. A possible reason the SVZa cells did not provoke an immune rejection by the host tissue could be because the SVZa cells used for transplantation were a substantially homogeneous population of neuronal progenitor cells. Neurons do not have antigen presenting capability and thus are not able to initiate an immune response. Glial cells,

the early targets in a rejection process, are generally absent from the transplanted SVZa cell suspension.

Appearance and distribution of SVZa cells restricted to the striatal boundary.

Even though similar coordinates were used for implantation in all the animals, the distribution of transplanted SVZa cells varied. In some cases (3 out of 12) following transplantation, the PKH26- or BrdU-labeled cells were identified only along the striatal boundary adjacent to the corpus callosum and not within the striatum proper (Table 1). Labeled SVZa cells were present along the dorsal, lateral and ventro-lateral aspects of the striatal boundary 2-4 weeks after implantation. The outlining of the contour of the striatum by labeled cells suggests that they had arrived at their position by migration, rather than being placed at the borders of the striatum simply as a result of the injection. Various intensities of BrdU staining was observed among the labeled SVZa cells, which were observed either individually or in small groups. The PKH26-labeled cells seen along the striatal boundary did not appear to have any prominent morphological features; they were often round without any processes similar to other individual cells. This indicates that the cells at the border of the striatum may not undergo differentiation as they do when situated in the striatum.

Appearance and distribution of SVZa cells within the striatum and along the striatal boundary.

In 5 out of the 12 animals labeled cells were seen both within the striatum and along the striatal boundary (Table 1) 2-4 weeks following transplantation. Also various intensities of BrdU staining were observed amongst the labeled cells. In the majority of the cases the SVZa cells located within the striatum, were in closer proximity to the striatal boundary and labeled SVZa cells were distributed all along the striatal edge between the striatum and the corpus callosum as described previously.

The relationship of the transplanted SVZa cells to the lateral cortical stream.

Of significance is the fact that in 8 of the 12 animals (67%) the SVZa cells were present along the striatal boundary. This region along the striatal boundary corresponds

to the lateral cortical stream of migration described by Bayer and Altman in *Neocortical Development*, New York:Raven Press, Ltd., pp. 116-127 (1991) which is present prenatally and is used by ventricular zone-derived cells of the developing cortex to reach the lateral and ventro-lateral cortical plate. The presence of transplanted SVZa cells distributed along this curved pathway, suggests that the SVZa cells are able to decipher guidance cues, used by other migrating cells.

Short Term Survival of Transplanted Neonatal Subventricular Zone Progenitor Cells.

The short-term behavior and phenotype of dissociated, BrdU-labeled SVZa cells transplanted stereotaxically into the striatum of adult rats was examined. Three days after transplantation most SVZa cells were immunoreactive for TuJ1, an antibody which recognizes neuron-specific class III β-tubulin. Within the adult striatum only the transplanted SVZa cells stained intensely for TuJ1. Three days after transplantation, TuJ1(+) cells were also identified within 50 - 250 μm of the transplant, suggesting that these cells had migrated from their site of implantation. Within two weeks, transplanted cells had dispersed up to 600 μm. A very small number of the transplanted cells were GFAP(+). However, the transplant contained numerous GABA(+) cells. Some of the transplanted cells, within the first couple of weeks of transplantation, were tyrosine hydroxylase positive, as determined by antibody staining. Thus, SVZa cells have the capability to disperse and differentiate into neurons following transplantation into an adult striatum.

Example 9

Transfection of neuronal progenitor cells: Cells were harvested from the SVZa, dissociated, and plated in 16 well chamber slides in Ham's F10 medium with 1% penicillin/streptomycin and 10% fetal calf serum. Between 3 x 10^4 and 8 x 10^4 cells per well were added. Either the next day or several hours later, the cells were infected with retrovirus (either BAG, which expresses βgal in the cytoplasm at 1.04 x 10^6 particles/ml, or nls-lacZ retroviral vector, which expresses βgal in the nucleus [gift of Dr. Gary Nolan; *Proc. Natl. Acad. Sci. USA* 84:6795-6799 (1987)], at 1.54 x 10^6 particles/ml) in varying amounts (30 μl-200 μl) and 0.6 μl/well of a 1 mg/ml solution of

polybrene was added. Cells were fixed a day later with 2% paraformaldehyde, 0.4% glutaraldehyde, 0.1 M PBS. The X-Gal incubation mixture (Luskin, *Neuron* 11:173 (1993)) was added and the number of blue cells/total cells in each dish was determined. Up to 4 % of the cells were blue, indicating they had been transfected or had inherited the transfected gene.

Example 10

Generation of immortalized clonal cell lines from the SVZa: Primary cultures can be made at low density from dissociated SVZa from newborn rats. These cultures can then be transfected with a retrovirus containing both the temperature sensitive SV40 Large T and neo^r genes. After the infection, G418 (a neomycin analog) can be added to the growth medium in order to select for cells that have integrated the retrovirus thus acquiring neomycin resistance. G418 selection can be maintained until colonies form on the dishes. After these colonies form, each can be isolated and expanded in separate dishes to produce sublines hopefully consisting of mitotic clones of a single infected primary cell.

Southern analysis can be used to verify or disprove the clonality of each subline. It is important to establish clonal cell lines due to the random nature of retroviral integration which may affect expression of the immortalizing Large T antigen. The SV40 Large T antigen cDNA can be used to probe several different restriction digests of genomic DNA isolated from each cell line. This can allow analysis of each subline for the length of the integrated construct, number of integration sites, and the clonal relationships between each line.

At the same time, each subline can be expanded in culture to demonstrate the ability to passage *in vitro*. As soon as enough cells are available, each subline can be frozen in order to preserve samples early in their immortalized life span.

To obtain cells from the SVZa, newborn (P0) Sprague-Dawley rat pups anesthetized by hypothermia can be decapitated, and the brains can be dissected into ice-cold Ca^{2+}/Mg2+ free HBBS. After removal of meninges, the anterior portion of the subventricular zone can be dissected under the microscope (Figure 1). This tissue can then be incubated in 0.15% trypsin in Eagle's Basal Medium for 20 minutes. Following

this incubation, the tissue can undergo aspiration with a fire-polished Pasteur pipette to generate a single cell suspension. Cells can then be plated at a low density in 1:1 DMEM:HAMS media supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin onto several poly-D-lysine coated 35 mm plastic culture dishes. The cells can be grown at 39°C for 24 hours.

Twenty-four hours after plating the primary SVZa cultures, the cells can be moved to 33°C, and the media can be replaced with the supernatant from the producer cell line containing the replication defective retrovirus encoding the ts SV40 Large T antigen. 8 μg/ml polybrene can also be added to the cultures to facilitate retroviral entry into the cells. After 4 hours, the retroviral supernatant can be replaced with fresh DMEM/HAMS medium, and the cells can be kept at 33°C. The following day, 0.5 mg/ml G418, a neomycin analog, can be added to the media in order to select for neomycin resistant cells. This selection media can be changed every 3-5 days. As colonies form on the dishes, they can be isolated with cloning rings and transferred to separate wells in a 24 well plate. Each subline can then be expanded and passaged to provide cells for study. A subset of each line can also be frozen in 10% DMSO in medium.

High molecular weight genomic DNA can be prepared from each cell line as previously described (Maniatis et al., *Molecular Cloning (A Laboratory Manual)*, Cold Spring Harbor, Cold Spring Laboratories, 1982). 10 μg of DNA can be cut with Xbal, EcoRI, and BgIII in separate reactions. Xbal cuts at both ends of the retroviral insert while both EcoRI and BgIII cut only once within the construct. Then, the DNA can be size fractionated on 0.8% agarose gels alongside DNA markers of known size and transferred to a nylon filter (GeneScreen Plus, Dupont) as described by Southern, 1975. The filterbound DNA can then be hybridized to a random primed SV40 Large T antigen probe under stringent conditions.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

5

What is claimed is:

1. An isolated cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which can give rise to progeny which can differentiate into neuronal cells.

2. The composition of Claim 1, wherein greater than about 95% of the mammalian, non tumor-derived, neuronal progenitor cells express a neuronal marker and can give rise to progeny which can differentiate into neuronal cells.

3. The composition of Claim 1, wherein the isolated neuronal progenitor cells can, without being first immortalized, divide for at least two generations.

4. The composition of Claim 1, wherein the neuronal progenitor cells are rat cells.

5. The composition of Claim 1, wherein the neuronal progenitor cells are human cells.

6. The composition of Claim 1, wherein at least a portion of the population of neuronal progenitor cells, or their progeny, is transfected with an exogenous nucleic acid.

7. The composition of Claim 6, wherein the exogenous nucleic acid functionally encodes a biologically active molecule.

8. The composition of Claim 7, wherein the exogenous nucleic acid functionally encodes a biologically active molecule that stimulates cell division or differentiation.

9. The composition of Claim 7, wherein the exogenous nucleic acid functionally encodes a biologically active molecule that functions in the synthesis of a neurotransmitter.

10. A method of delivering a biologically active molecule produced by the neuronal progenitor cells of the cellular composition of Claim 1, or their progeny, or mixtures thereof, to a region of a mammalian brain comprising transplanting the cellular composition of Claim 1 into the region of the brain, thereby delivering a biologically active molecule produced by the cells or their progeny to the region.

11. A method of delivering a biologically active molecule produced by the neuronal progenitor cells of the cellular composition of Claim 7, or their progeny, or mixtures thereof, to a region of a mammalian brain comprising transplanting the cellular composition of Claim 7 into the region of the brain, thereby delivering the biologically active molecule produced by the cells or their progeny to the region.

12. A method of treating a neuronal disorder characterized by a reduction of catecholamines in the brain of a mammal, comprising transplanting into the brain the cellular composition of Claim 1, or their progeny, or mixtures thereof, thereby providing a source of catecholamines to the brain and treating the disorder.

13. The method of Claim 12, wherein the neuronal disorder is Parkinson's disease.

14. A method of treating Alzheimer's disease in a subject comprising transplanting into the brain of the subject the cellular composition of Claim 8, or their progeny, or mixtures thereof, thereby treating Alzheimer's disease.

15. A method of treating Alzheimer's disease in a subject comprising transplanting into the brain of the subject the cellular composition of Claim 9, or their progeny, or mixtures thereof, thereby treating Alzheimer's disease.

16. A method of treating a neuronal disorder characterized by a reduction of γ-aminobutyric acid in the brain in a mammal, comprising transplanting into the brain the cellular composition of Claim 1, or their progeny, or mixtures thereof, thereby providing a source of γ-aminobutyric acid to the brain and treating the disorder.

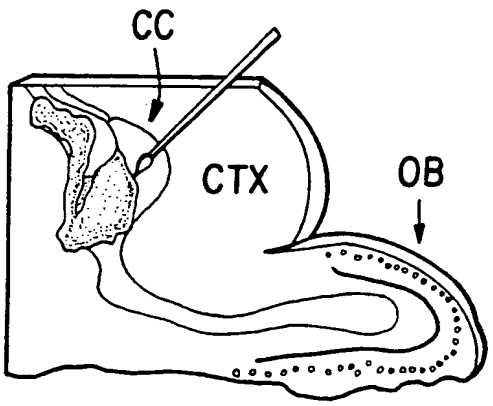
17. The method of Claim 16, wherein the neuronal disorder is Huntington's Disease.

18. A method of screening for a marker of neuronal cells comprising obtaining the neuronal progenitor cells of Claim 1, and detecting the presence of a marker in the neuronal progenitor cells that is not present in non-neuronal cells, the marker present in the neuronal progenitor cells that is not present in the non-neuronal cells being a marker of neuronal cells.

19. A method of detecting a neuronally expressed gene comprising obtaining a cDNA library from the neuronal progenitor cells of Claim 1, obtaining a cDNA library from a non-neuronal cell, determining the presence at higher levels of a cDNA in the library from the neuronal progenitor cells than in the non-neuronal cell, the presence at higher levels of a cDNA in the library from the neuronal progenitor cells indicating a neuronally expressed gene.

20. A method of obtaining an isolated cellular composition comprising greater than about 90% mammalian, non tumor-derived, neuronal progenitor cells which express a neuronal marker and which can give rise to progeny which can differentiate into neuronal cells, comprising isolating cells from the portion of a mammalian brain that is the equivalent of the anterior portion of the subventricular zone at the dorsolateral portion of the anterior-most extent of the region surrounding the ventricle of a neonatal rat brain and culturing the isolated cells in the absence of mitotic inhibitors.

21. An isolated cellular composition comprising greater than about 50% mammalian, non tumor-derived, neuronal progenitor cells which express a neuron-specific marker and which give rise to progeny which can differentiate into neuronal cells.



DISSECTION

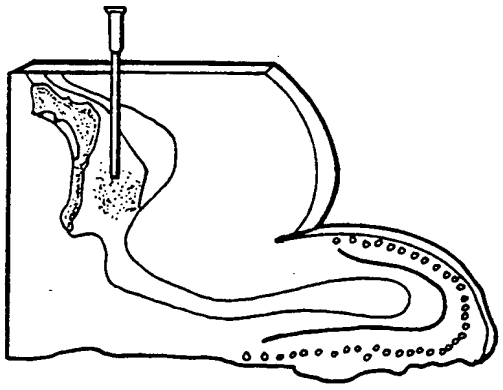
FIG. 1A

DISSOCIATION

FIG. 1B

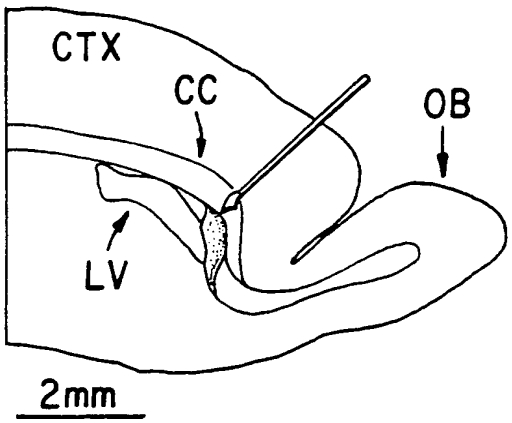
LABELING

FIG. 1C



TRANSPLANTATION

FIG. 1D



DISSECTION

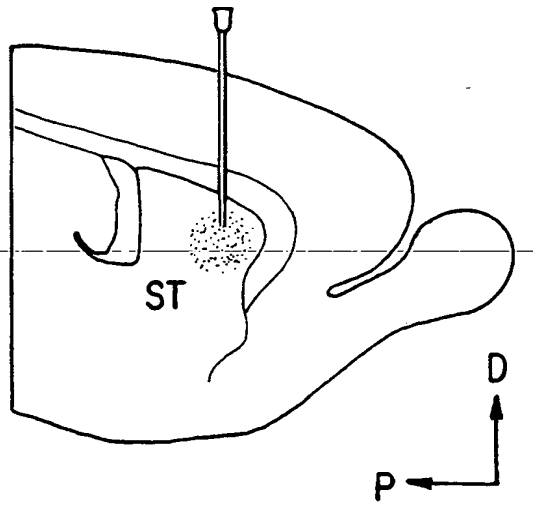
FIG. 2A

DISSOCIATION

FIG. 2B

LABELING

FIG. 2C



TRANSPLANTATION

FIG. 2D

INTERNATIONAL SEARCH REPORT

[redacted]national application No.
PCT/US96/11304

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C12N 5/00; C12Q 1/02, 1/68
US CL :435/6, 29, 240.1; 424/93.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 29, 240.1; 424/93.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	REYNOLDS, B. A. et al. Generation of Neurons and Astrocytes from Isolated Cells of the Adult Mammalian Central Nervous System. Science. 27 March 1992, Vol. 255, pages 1707-1710, especially page 1708.	1-9, 20 and 21
Y	US 5,082,670 A (F. GAGE ET AL) 21 January 1992, column 2, line 32 to column 3, line 25 and column 23, line 30 to column 26, line 3.	10-17
Y	VAYSSE, P. J-J. et al. A Clonal Analysis of Glial Lineages in Neonatal Forebrain Development In Vitro. Neuron. September 1990, Vol. 5, pages 227-235, especially page 228 and 229.	18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 SEPTEMBER 1996	Date of mailing of the international search report 0 2 OCT 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DEBORAH CROUCH Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

PCT /ELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 6 :		(11) Internationale Veröffentlichungsnummer: WO 99/32606
C12N 5/00	A2	(43) Internationales Veröffentlichungsdatum: 1. Juli 1999 (01.07.99)

(21) Internationales Aktenzeichen: PCT/DE98/03817

(22) Internationales Anmeldedatum: 18. Dezember 1998 (18.12.98)

(30) Prioritätsdaten:
197 56 864.5 19. Dezember 1997 (19.12.97) DE

(71)(72) Anmelder und Erfinder: BRÜSTLE, Oliver [DE/DE]; Lindenweg 17, D–53340 Meckenheim (DE).

(74) Anwälte: VOSSIUS, Volker usw.; Holbeinstrasse 5, D–81679 München (DE).

(81) Bestimmungsstaaten: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht
Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.

(54) Title: NEURAL PRECURSOR CELLS, METHOD FOR THE PRODUCTION AND USE THEREOF IN NEURAL DEFECT THERAPY

(54) Bezeichnung: NEURALE VORLÄUFERZELLEN, VERFAHREN ZU IHRER HERSTELLUNG UND IHRE VERWENDUNG ZUR THERAPIE VON NEURALEN DEFEKTEN

(57) Abstract

The invention relates to isolated and purified neural precursor cells, to a method for the production thereof from embryonal stem cells in unlimited quantities, to the use of neural precursor cells in neural defect therapy, especially in mammals, preferably human beings, and to obtain polypeptides.

(57) Zusammenfassung

Die Erfindung betrifft isolierte und gereinigte neurale Vorläuferzellen, Verfahren zu ihrer Herstellung aus embryonalen Stammzellen in unbegrenzter Menge, die Verwendung der neuralen Vorläuferzellen zur Therapie von neuralen Defekten insbesondere bei Säugern, vorzugsweise Menschen und zur Gewinnung von Polypeptiden.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AL	Albanien	**ES**	Spanien	**LS**	Lesotho	**SI**	Slowenien
AM	Armenien	**FI**	Finnland	**LT**	Litauen	**SK**	Slowakei
AT	Österreich	**FR**	Frankreich	**LU**	Luxemburg	**SN**	Senegal
AU	Australien	**GA**	Gabun	**LV**	Lettland	**SZ**	Swasiland
AZ	Aserbaidschan	**GB**	Vereinigtes Königreich	**MC**	Monaco	**TD**	Tschad
BA	Bosnien-Herzegowina	**GE**	Georgien	**MD**	Republik Moldau	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagaskar	**TJ**	Tadschikistan
BE	Belgien	**GN**	Guinea	**MK**	Die ehemalige jugoslawische Republik Mazedonien	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Griechenland			**TR**	Türkei
BG	Bulgarien	**HU**	Ungarn	**ML**	Mali	**TT**	Trinidad und Tobago
BJ	Benin	**IE**	Irland	**MN**	Mongolei	**UA**	Ukraine
BR	Brasilien	**IL**	Israel	**MR**	Mauretanien	**UG**	Uganda
BY	Belarus	**IS**	Island	**MW**	Malawi	**US**	Vereinigte Staaten von Amerika
CA	Kanada	**IT**	Italien	**MX**	Mexiko		
CF	Zentralafrikanische Republik	**JP**	Japan	**NE**	Niger	**UZ**	Usbekistan
CG	Kongo	**KE**	Kenia	**NL**	Niederlande	**VN**	Vietnam
CH	Schweiz	**KG**	Kirgisistan	**NO**	Norwegen	**YU**	Jugoslawien
CI	Côte d'Ivoire	**KP**	Demokratische Volksrepublik Korea	**NZ**	Neuseeland	**ZW**	Zimbabwe
CM	Kamerun			**PL**	Polen		
CN	China	**KR**	Republik Korea	**PT**	Portugal		
CU	Kuba	**KZ**	Kasachstan	**RO**	Rumänien		
CZ	Tschechische Republik	**LC**	St. Lucia	**RU**	Russische Föderation		
DE	Deutschland	**LI**	Liechtenstein	**SD**	Sudan		
DK	Dänemark	**LK**	Sri Lanka	**SE**	Schweden		
EE	Estland	**LR**	Liberia	**SG**	Singapur		

Neurale Vorläuferzellen, Verfahren zu ihrer Herstellung und ihre Verwendung zur Therapie von neuralen Defekten

Die Erfindung betrifft isolierte und gereinigte neurale Vorläuferzellen aus embryonalen Stammzellen (ES-Zellen), Verfahren zu ihrer Herstellung in unbegrenzter Menge, die Verwendung der neuralen Vorläuferzellen zur Therapie von neuralen Defekten, insbesondere bei Säugern, vorzugsweise Menschen und zur Gewinnung von Polypeptiden.

Die Transplantation von Hirnzellen in das Nervensystem von Säugern stellt eine erfolgversprechende Methode für die Behandlung zahlreicher neurologischer Erkrankungen dar. In Tierversuchen wurden bereits zahlreiche Zellpopulationen in Gehirn und Rückenmark transplantiert (Björklund, aus: Molecular and Cellular Approaches to the Treatment of Neurological Disease, Raven Press, New York, 1993; Brüstle & McKay, Curr. Opinion Neurobiol. 6:688-695, 1996). In jüngster Zeit wurde die neurale Transplantation auch an selektierten Fällen klinisch eingesetzt, beispielsweise bei Patienten, welche an der Parkinson'schen Erkrankung leiden (Lindvall, aus: Neural transplantation in Parkinson's disease, Raven Press, New York, 1994; Olanow et al., TINS 19:102-109, 1996).

Im Gegensatz zu vielen anderen Organen zeigt das ausgereifte Nervensystem der Säuger eine außerordentlich geringe Regenerationsfähigkeit. Dies liegt im wesentlichen daran, daß die für den Aufbau des Nervensystems notwendigen Vorläuferzellen mit einigen wenigen Ausnahmen nur während der Hirnentwicklung vorhanden sind. Gerade solche unreifen Vorläuferzellen sind jedoch erforderlich, um Defekte im ausgereiften Nervensystem durch Transplantation wiederherzustellen. Dies hat zur Folge, daß als Donorgewebe für neurale Transplantate vorwiegend aus dem embryonalen Gehirn gewonnene Zellen benützt werden. So muß beispielsweise derzeit Hirngewebe von bis zu sieben menschlichen Embryonen gepoolt werden, um genügend Material für die Transplantation eines Parkinson-Patienten zu erhalten. Dies wirft nicht nur enorme ethische Probleme auf. Es ist anzunehmen, daß ein solches Verfahren auch bei Inkaufnehmen der ethischen Problematik nicht in dem Umfang umsetzbar ist, wie es für die

Aufrechterhaltung einer der breiten Öffentlichkeit zugänglichen zelltherapeutischen Behandlungsstrategie erforderlich wäre.

In jüngster Zeit sind mehrere Versuche unternommen worden, die begrenzte Verfügbarkeit von embryonalen Hirnzellen von Säugern durch eine der Transplantation vorgeschaltete Vermehrung der Vorläuferzellen *in vitro* zu kompensieren. Hierbei wurden im wesentlichen zwei Strategien verfolgt. Eine Methode besteht darin, die Vorläuferzellen mit Hilfe sogenannter Onkogene zu immortalisieren. Hierbei wird ein zumeist ursprünglich aus Tumoren isoliertes Gen in das Genom der Zellen inseriert. Dieses „Tumorgen" veranlaßt die Zellen zu kontinuierlichem und in der Regel unkontrolliertem Wachstum (Lendahl & McKay, TINS 13:132-137, 1990).

Neuere und besser steuerbare Varianten dieser Methode bestehen darin, sogenannte temperatur-sensitive Mutanten zu benützen. In diesem Fall können die Zellen unter einer "permissiven" Temperatur *in vitro* vermehrt werden. Wird die nicht-permissive Temperatur entsprechend der Körpertemperatur des Empfängers gewählt, ist das Genprodukt nach Transplantation nicht mehr stabil und es kommt zum Wachstumsstop (Renfranz et al., Cell 66:713-729, 1991). Allerdings verbleibt das Onkogen in den transplantierten Zellen. Eine mögliche Schwellenaktivität oder spätere Reaktivierung kann bei diesem Verfahren nicht vollständig ausgeschlossen werden. Neurere Bemühungen gehen deshalb dahin, das Onkogen durch eine molekularbiologische Methode nach der Zellvermehrungsphase wieder aus dem Genom der Vorläuferzellen zu entfernen (Westerman & Leboulch, Proc. Natl. Acad. Sci. USA 93:8971-8976, 1996). Wie alle Zelllinien besitzen auch die Onkogen-immortalisierten Vorläuferzellen ein hohes Risiko für chromosomale Aberationen.

Eine weitere Methode zur *in vitro* Vermehrung von Vorläuferzellen vor einer Transplantation besteht darin, die aus dem Gehirn entnommenen Zellen mit Wachstumsfaktoren zu behandeln (Cattaneo & McKay, Nature 347:762-765, 1990; Reynolds & Weiss, Science 255:1707-1710, 1992; Richards et al., Proc. Natl. Acad. Sci. USA 89:8591-8595, 1992; Ray et al., Proc. Natl. Acad. Sci. USA 90:3602-3606, 1993; Kilpatrick & Bartlett, Neuron 10:255-265, 1993; Ray & Gage, J. Neurosci. 6:3548-3564, 1994; Davis & Temple, Nature 372:263-266, 1994; Vicario-Abej—n et al., Neuron 15:105-114, 1995; Gosh & Greenberg, Neuron 15:89-103, 1995; Gritti et al., J. Neurosci. 16:1091-1100, 1996). Zur Zeit kann nicht abschließend beurteilt werden, in

welchem Umfang eine *in vitro* Vermehrung von Vorläuferzellen durch Wachstumsfaktorbehandlung möglich ist. Erste Transplantionsversuche mit derartig behandelten Zellen lieferten widersprüchliche Ergebnisse. Während manche Wissenschaftler eine mangelhafte Integrationsfähigkeit feststellten (Svendsen et al., Exp. Neurol. 137:376-388, 1996), scheinen andere Experimente auf eine Inkorporation solcher Zellen ins Empfängergehirn hinzuweisen (Gage et al., Proc. Natl. Acad. Sci. USA 92:11879-11883, 1995).

Zusammenfassend läßt sich bezüglich dieser Zellvermehrungsstrategien sagen, daß der mögliche Umfang einer Wachstumsfaktor-vermittelten *in vitro* Vermehrung unklar und das Verhalten derartig expandierter Zellen nach Transplantation nicht eindeutig geklärt ist. Onkogen-vermittelte Zellvermehrungsstrategien beinhalten hohe Risiken seitens des eingeführten Onkogens und etwaiger chromosomaler Aberrationen. Der schwerwiegendste Nachteil beider Strategien ist jedoch die Tatsache, daß als Anfangsmaterial Hirngewebe erforderlich ist, welches vorwiegend embryonalen Spendergehirnen entnommen wird.

Embryonale Stammzellen (ES-Zellen) bieten eine völlig neue Perspektive für die Herstellung von Donorzellen für Transplantationszwecke. Derartige Zellen wurden erstmals 1981 in der Maus beschrieben (Martin, Proc. Natl. Acad. Sci. USA 78:7634-7638, 1981; Evans & Kaufman, Nature 292:154-156, 1981). Es handelt sich um Zellen, welche beispielsweise bei der Maus aus der sog. inneren Zellmasse von etwa 3,5 Tage alten Embryonen gewonnen werden. Diese Zellen sind totipotent und können in alle Zell- und Gewebstypen ausdifferenzieren. Der augenfälligste Beweis hierfür ist die Tatasche, daß in einen anderen Embryo injizierte ES-Zellen in der Lage sind, sich an der Bildung aller Gewebe - einschließlich der Keimbahnpopulationen - zu beteiligen und sogenannte chimäre Tiere zu erzeugen (Bradley et al., Nature 309:255-256, 1984). Das einzigartige an ES-Zellen ist, daß sie sich in Anwesenheit von leukemia inhibitory factor (LIF) über viele Passagen in ihrem totipotenten Zustand halten und vermehren lassen (Smith et al., Nature 336:688-690, 1988). Dies wird heute vielfach dafür benutzt, die Zellen *in vitro* genetisch zu modifizieren, um dann nach Injektion dieser Zellen in eine Blastozyste genetisch veränderte Tiere herzustellen (Robertson et al., Nature 323:445-448, 1986). Ein weitaus weniger häufig begangener Weg ist die *in vitro* Differenzierung von ES-Zellen. Diese Technik erlaubt es u.a., die frühen Schritte der Gewebeentwicklung unter kontrollierten Bedingungen *in vitro* ablaufen zu lassen und

experimentell zu untersuchen. ES-Zellen sind mittlerweile aus den verschiedensten Spezies isoliert worden, so z.B. aus Ratten (Iannaconne et al., Dev. Biol. 163:288-292, 1994), Hamstern (Doetschman et al., Dev. Biol. 127:224-227, 1988), Vögeln (Pain et al., Development 122:2339-2348, 1996), Fischen (Sun et al., Mol. Mar. Biol. Biotechno. 4:193-199, 1995), Schweinen (Wheeler, Reprod. Fertil. Dev. 6:563-568, 1994), Rindern (First et al., Reprod. Fertil. Dev. 6:553-562) und Primaten (Thomson et al., Proc. Natl. Acad. Sci. USA 92:7844-7848, 1995). Mehrere Monate nach Einreichung der prioritätsbegründenden deutschen Patentanmeldung Nr. 197 56 864.5 gelang es zwei Forschergruppen, ES Zellen bzw. ES-Zell-ähnliche Stammzellen aus menschlichem Embryonalgewebe zu isolieren (Thomson et al., Science 282: 1145-1147, 1998; Shamblott et al., Proc. Natl. Acad. Sci. USA 95: 13726-13731, 1998). Neuere Studien weisen außerdem darauf hin, daß Embryonen und damit ES-Zellen auch durch Transplantation von aus embryonalen und reifen Säugetierzellen gewonnenen Zellkernen in enukleierte Eizellen (Oocyten) generiert werden können (Campbell et al., Nature 380:64-66, 1996; Wilmut et al., Nature 385:810-813, 1997).

Während der vergangenen Jahre ist es mehreren Forschungsgruppen gelungen, ES-Zellen *in vitro* in Zellen des Nervensystem auszudifferenzieren. In der überwiegenden Zahl der Fälle wurde dies durch Zugabe von Retinsäure zu aggregierten ES-Zellen erreicht (Bain et al., Dev. Biol. 168:342-357, 1995; Strübing et al., Mech. Dev. 53:275-287, 1995; Fraichard et al., J. Cell Sci. 108:3181-3188, 1995; Finley et al., J. Neurosci. 16:1056-1065, 1996). Einige der unter diesen Bedingungen differenzierenden Zellen wiesen neuronale (Bain et al., Dev. Biol. 168:342-357, 1995; Strübing et al., Mech. Dev. 53:275-287, 1995; Fraichard et al., J. Cell Sci. 108:3181-3188, 1995; Finley et al., J. Neurosci. 16:1056-1065, 1996) und gliale (Fraichard et al., J. Cell Sci. 108:3181-3188, 1995) Eigenschaften auf. Die Retinsäure-vermittelte Induktion einer neuralen Differenzierung hat zwei wesentliche Nachteile. Zum einen erfolgt eine neurale Differenzierung nur in einem Teil der Zellen. Eine befriedigende Aufreinigung dieser Zellen war bisher nicht möglich. Zum anderen übt Retinsäure einen starken differenzierenden Einfluß auf die ES-Zellen aus. Die in Anwesenheit von Retinsäure differenzierten Neuronen und Gliazellen haben zum ganz überwiegenden Teil das Vorläuferzellstadium bereits überschritten und befinden sich vielfach in einem postmitotischen Stadium. Einer Verwendung zur Transplantation und einer weiteren Anreicherung dieser Zellen sind somit Grenzen gesetzt.

Kürzlich wurde eine alternative Methode zur Gewinnung von neuralen Vorläuferzellen aus ES-Zellen beschrieben (Okabe et al., Mech. Dev. 59:89-102, 1996). Hierbei werden die zu sog. Embryoid Bodies aggregierten ES-Zellen in serumfreiem Medium ausplattiert und über mehrere Tage kultiviert. Währed dieser Zeit kommt es zu massivem Zelltod insbesondere innerhalb der nicht-neuralen Zellen. Am Ende dieser Phase zeigen mehr als 80% der Zellen eine Expression von Nestin, ein Intermediärfilament, das typischerweise in neuralen Vorläuferzellen gebildet wird (Frederiksen & McKay, J. Neurosci. 8:1144-1151, 1988; Lendahl et al., Cell 60:585-595, 1990). Diese Vorläuferzellen sind mit Hilfe von basischem Fibroblastenwachstumsfaktor (bFGF) als Zellrasen weiter vermehrbar und differenzieren nach Entzug von bFGF in Neurone und Astrozyten aus (Okabe et al., Mech. Dev. 59:89-102, 1996). Die Expansionsfähigkeit unter bFGF Gabe ist allerdings limitiert; bereits nach wenigen Passagen kommt es zu einer zunehmenden astrozytären Differenzierung der Zellen (Okabe, aus: Current Protocols in Neuroscience, John Wiley, New York, 1997). Nach einer derartig limitierten Wachstumsphase sind innerhalb der Kulturen immer noch zahlreiche undifferenzierte embryonale und nicht-neural differenzierte Zellen vorhanden. Zellpopulationen mit derartigen Kontaminationen sind für mögliche rekonstruktive Transplantationen nicht geeignet, da undifferenzierte ES-Zellen zu Tumoren (Teratokarzinome) führen können und nicht-neural differenzierte Donor-Zellen zur Ausbildung nicht-neuraler Gewebe im Transplantat führen. Bisher ist kein Verfahren bekannt, mit dem es möglich wäre, aus ES-Zellen Zellen mit neuronalen oder glialen Eigenschaften in einer Reinheit herzustellen, die eine Transplantation ohne Tumorbildung in das Nervensystem und eine funktionelle Aktivität in vivo, wie z.B. eine Remyelinisierung oder ein Nervenzellersatz mit Normalisierung des durch den Nervenzellverlust bedingten abnormen Verhaltens erlaubt.

Die Bereitstellung ausreichender Mengen definierter neuraler Vorläuferzellen stellt nach dem gegenwärtigen Stand der Technik eines der Hauptprobleme für die Transplantation von Hirnzellen dar. Zur Zeit werden solche Vorläuferzellen aus embryonalem Hirngewebe von Säugern gewonnen, wobei beispielsweise für die Transplantation eines Parkinson-Patienten Material von bis zu sieben menschlichen Embryonen erforderlich ist. Eine solche Strategie ist ethisch äußerst problematisch und keinesfalls imstande, die Behandlung einer größeren Zahl von Parkinson-Patienten auf Dauer zu gewährleisten. Bemühungen, die entnommenen Hirnzellen vor der Transplantation *in vitro* zu vermehren, haben bislang zu keinen signifikanten Verbesserungen geführt. Onkogen-vermittelte Immortalisierungsversuche sind aufgrund der

Einführung eines Onkogens in die zu transplantierenden Zellen sehr riskant. Der Umfang einer möglichen Wachstumsfaktor-vermittelten Vermehrung von Vorläuferzellen ist bislang nicht signifikant. Weiterhin herrscht Skepsis über das Inkorporationsvermögen solcher Zellen nach Transplantation (Svendsen et al., Exp. Neurol. 137:376-388, 1996).

ES-Zellen stellen eine interessante alternative Donorquelle für neurale Transplantate dar. Dies liegt daran, daß sich diese Zellen über lange Zeit in einem undifferenzierten, totipotenten Zustand zu großen Zellmengen vermehren lassen (Slack, aus: From Egg to Embryo, Cambridge University Press, Cambridge, 1991). Dabei behalten sie die Fähigkeit bei, in alle Gewebe, auch in Hirngewebe, auszudifferenzieren. Allerdings war es bisher nicht möglich, selektiv neurale Vorläuferzellen aus ES-Zellen herzustellen. Versuche, ES-Zellen mit Hilfe von Retinsäure in neurale Zellen zu differenzieren (Bain et al., Dev. Biol. 168:342-357, 1995; Strübing et al., Mech. Dev. 53:275-287, 1995; Fraichard et al., J. Cell Sci. 108:3181-3188, 1995; Finley et al., J. Neurosci. 16:1056-1065, 1996) ergaben stets gemischte Zellpopulationen, in denen neurale Zellen nur einen kleinen Teil der Zellen ausmachten. Weiter wurden bisher mit Retinsäure zwar Neurone aber keine neuralen Vorläuferzellen generiert. In einer Arbeit von Dinsmore *et al.*, Cell Transplant. 5: 131-143 (1996), wurden derartige gemischte Populationen ins Gehirn Quinolinsäure-behandelter Ratten transplantiert. Quinolinsäure ist ein bekanntes Neurotoxin, das Nervenzellen schädigt und zerstört. Nach Transplantation behielten einige der Zellen ihren neuronalen Phänotyp bei. Eine funktionelle Innervation des Empfängergehirns durch diese Zellen oder gar eine Wiederherstellung verlorengegangener Hirnfunktion wurden bei diesen Experimenten nicht beobachtet (Dinsmore et al., Cell Transplant. 5:131-143, 1996). Die von Okabe geschilderte Kultivierung plattierter Embryoid Bodies in ITSFn Medium erfolgt ohne Retinsäure und liefert bis zu 85% Zellen, welche den Vorläuferzell-Marker Nestin exprimieren (Okabe et al., Mech. Dev. 59:89-102, 1996). Allerdings ist auch diese Population nicht genügend rein, um sie für rekonstruktive Zwecke zu benützen. So bilden beispielsweise in ITSFn kultivierte ES-Zellen nach Transplantation primitive neuroepitheliale Strukturen und sogar nicht-neurales Gewebe wie z.B. Knorpel und Drüsengewebe. Eine weitere Proliferation der in ITSFn kultivierten Zellen in Anwesenheit von bFGF ist möglich. Allerdings verlieren die Zellen rasch ihre Multipotenz und differenzieren bereits nach wenigen Passagen überwiegend in Astrozyten aus. Die nicht neural differenzierten Zellen konnten während dieser kurzen Proliferationsphase bisher nicht von den neuralen Vorläuferzellen abgetrennt werden. Ein weiterer schwerwiegender Nachteil dieses

Systems ist, daß es keine effiziente Produktion von Oligodendrozyten erlaubt. So fanden Okabe et al. nach Wachstumsfaktorentzug-vermittelter Differenzierung allein keine Oligodendrozyten, und selbst nach zusätzlicher Gabe des Hormons T3 ließ sich nur in 1-2% der Zellen eine Expression oligodendroglialer Antigene nachweisen (Okabe et al., Mech. Dev. 59:89-102, 1996). Was die neuronale Differenzierung anbelangt, wurden in den von Okabe et al. durchgeführten Arbeiten keine Neurone beschrieben, welche Tyrosinhydroxylase, Cholinacetyltranferase oder Serotonin produzieren, Stoffe, die für die Signalübertragung in Nervenzellen von großer Bedeutung sind. Weiter wurden in diesen Arbeiten keine neuralen Zellen erhalten, welche Peripherin produzieren. Peripherin wird typischerweise von peripheren Neuronen und Neuronen in Hirnstamm und Rückenmark produziert.

Der Erfindung liegt daher die Aufgabe zugrunde, isolierte, gereinigte nicht-tumorigene Vorläuferzellen mit neuronalen oder glialen Eigenschaften aus embryonalen Stammzellen (ES-Zellen), insbesondere gereinigte Neurone und Gliazellen, sowie Verfahren zur Herstellung der Vorläuferzellen in praktisch unbegrenzter Menge bereitzustellen. Mit Hilfe dieser gereinigten Vorläuferzellen gelingt eine Transplantation ohne Tumorbildung in das Nervensystem und eine funktionelle Aktivität in vivo, wie z.B. eine Remyelinisierung oder ein Nervenzellersatz mit Normalisierung des durch den Nervenzellverlust bedingten abnormen Verhaltens, und eine Verbesserung der Therapie neuraler Defekte. Die Lösung dieser Aufgaben ergibt sich aus den Patentansprüchen, der nachfolgenden Beschreibung und den Abbildungen.

Definitionen

Der hier verwendete Begriff *Astrozyt* bedeutet eine Gliazelle des Nervensystems, über deren Funtion noch wenig bekannt ist. Die Fortsätze dieser Zellen bilden einen Teil der sog. Bluthirn-schranke, ein Trennsystem zwischen dem Blutkreislauf und den hirneigenen Flüssigkeiten. Der hier verwendete Begriff *autolog* bedeutet, daß die Zellen vom selben Individuum stammen. Der hier verwendete Begriff *bFGF* bedeutet "Basic Fibroblast Growth Factor". bFGF ist ein Wachstumsfaktor, der u.a. neurale Vorläuferzellen zur Teilung (Proliferation) anregt. Der hier verwendete Begriff *CNTF* bedeutet "Ciliary Neurotrophic Factor". Der hier verwendete Begriff *DMEM/F12* bedeutet Dulbecco's Modified Eagle Medium/Nutritient Mix F12 (1:1). DMEM/F12 ist ein kommerziell erhältliches, für die serumfreie Zellkultur verwendetes Medium und z.B. von Life Technologies, Nr. 11320 erhältlich. Der hier verwendete Begriff *EGF* bedeutet "Epidermal Growth Factor". EGF ist ein Wachstumsfaktor, der u.a. neurale

Vorläuferzellen zur Teilung (Proliferation) anregt. Der hier verwendete Begriff *Embryoid Bodies* bedeutet im Überstand schwimmende Zellaggregate, die von in Bakterienkulturschalen kultivierten ES-Zellen gebildet werden. Diese Zellaggregate zeigen Ähnlichkeit zu frühen Embryonen und bilden eine Vielzahl verschiedener Gewebe und werden deshalb als Embryoid Bodies bezeichnet. Der hier verwendete Begriff *ES-Zellen* bedeutet Embryonale Stammzellen. ES-Zellen können z.B. aus sehr frühen Embryonen von Säugern im Blastozystenstadium gewonnenen werden. Es handelt sich um totipotente Zellen, welche über viele Passagen in einem undifferenzierten Zustand gehalten werden können und die Fähigkeit besitzen, in alle Gewebe und Zelltypen auszudifferenzieren. ES-Zellen können auch durch Proliferation von Oocyten nach einer Kerntransplantation erhalten werden. Der Begriff ES-Zellen bedeutet ferner ES-Zell-ähnliche Zellen, die aus embryonalen Keimzellen (embryonic germ cells) erhalten werden. Der hier verwendete Begriff *Feeder-Zellen* bedeutet eine Zellpopulation, welche das Wachstum einer zu züchtenden Zellpopulation unterstützt. Beispielsweise werden ES-Zellen vielfach auf einem aus embryonalen Fibroblasten bestehenden Feeder-Zellrasen gezüchtet. Die hier verwendeten Begriffe *ITSFn* und *N3FL* sind Medien, die vom DMEM/F12-Medium abgewandelt sind (Okabe et al., Mech. Dev. 59:89-102, 1996). Der hier verwendete Begriff *LIF* bedeutet "Leukemia inhibitory factor". LIF ist ein Faktor, welcher die Differenzierung von ES-Zellen verhindert. Der hier verwendete Begriff *neurale Vorläuferzellen* bedeutet unreife Zellen, welche die Fähigkeit haben, reife Zellen des Nervensystems, z.B. Neurone (Nervenzellen) und Glia (Astrozyten und Oligodendrozyten) zu bilden. Der hier verwendete Begriff *neurale Sphäroide* bedeutet durch Proliferation neuraler Vorläuferzellen in serumfreien Wachstumfaktor-haltigem Medium in unbeschichteten Zellkulturschalen erhaltene Zellaggregate. Der hier verwendete Begriff *Oligodendrozyt* bedeutet eine Zelle des Nervensystems, die den sog. Gliazellen untergeordnet ist. Wichtigste bekannte Funktion dieser Zellen ist die elektrische Isolierung von Nervenzellfortsätzen (Axonen). Dabei werden die Axone von einer von den Oligodendrozyten gebildeten Hülle (Myelin) umhüllt. Schäden in der Myelinisierung führen zu sog. demyelinisierenden Erkrankungen. Eine der bekanntesten demyelinisierenden Erkrankungen ist die multiple Sklerose (MS). Der hier verwendete Begriff *PDGF* bedeutet "Platelet-Derived Growth Factor". PDGF ist ein Wachstumsfaktor, der u.a. in Kombination mit anderen Wachstumfaktoren neurale Vorläuferzellen zur Teilung (Proliferation) anregt. PDGF kommt in Form der Dimere AA, AB und BB vor und wird als *PDGF-AA*, *PDGF-AB* oder *PDGF-BB* bezeichnet. Der hier verwendete Begriff *toti-, pluri-, multi- und bipotente Zellen* bedeutet

Vorläuferzellen, welche in alle (totipotent), viele verschiedene (pluri- oder multipotent) oder zwei (bipotent) reife Zelltypen ausdifferenzieren können. In der Neurobiologie werden bipotente Zellen oft als Synonym für solche Vorläuferzellen verwendet, die noch in Astrozyten und Oligodendrozyten ausdifferenzieren können.

Somit betrifft die Erfindung isolierte, nach Transplantation in das Nervensystem nicht-tumorigene gereinigte Vorläuferzellen aus embryonalen Stammzellen (ES-Zellen) mit neuronalen oder glialen Eigenschaften, die vorzugsweise höchstens etwa 15% primitive embryonale und nicht-neurale Zellen enthalten. Bevorzugt sind Vorläuferzellen in Form eines Zellrasens oder Sphäroiden. Besonders bevorzugt sind Vorläuferzellen mit neuronalen, astrozytären und/oder oligodendroglialen Eigenschaften. Ferner bevorzugt sind Vorläuferzellen, die nach Proliferation von Oocyten, die durch Kerntransplantation hergestellt wurden, erhalten werden. Ferner bevorzugt sind Vorläuferzellen, die aus embryonalen Stammzellen erhalten werden, die ihrerseits aus embryonalen Keimzellen (embryonic germ cells) erhalten werden. Besonders bevorzugt sind Vorläuferzellen, die von Säuger-ES-Zellen oder Säuger-Oocyten erhalten werden. Insbesondere bevorzugt sind Vorläuferzellen der Maus, Ratte, Hamster, Schaf, Schwein, Rind, Primaten oder Mensch. Ferner besonders bevorzugt sind Vorläuferzellen, die gentechnisch modifiziert sind. Besonders bevorzugt sind Vorläuferzellen in tiefgefrorener Form. Ferner bevorzugt sind Zellbibliotheken, umfassend autologe und nicht autologe Vorläuferzellen.

Die Erfindung betrifft ferner ein Verfahren zur Herstellung der gereinigten Vorläuferzellen mit neuronalen oder glialen Eigenschaften, umfassend die folgenden Schritte: a) proliferieren von ES-Zellen, b) kultivieren der ES-Zellen aus Schritt a) zu neuralen Vorläuferzellen, c) proliferieren der neuralen Vorläuferzellen in einem Wachstumsfaktor-haltigen serumfreien Medium, d) proliferieren der neuralen Vorläuferzellen aus Schritt c) in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium und isolieren der gereinigten Vorläuferzellen und e) proliferieren der Vorläuferzellen aus Schritt d) in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium und isolieren der gereinigten Vorläuferzellen mit neuronalen oder glialen Eigenschaften. Bevorzugt ist eine Proliferation der ES-Zellen in Schritt a) zu Zellaggregaten, insbesondere Embryoid Bodies. Ferner bevorzugt ist ein Verfahren, wobei das Wachstumsfaktor-haltige serumfreie Medium in Schritt c) bFGF umfaßt. Ferner bevorzugt ist ein Verfahren, wobei das Wachstumsfaktor-haltige serumfreie Medium in Schritt d) bFGF und

EGF umfaßt. Ferner bevorzugt ist ein Verfahren, wobei das Wachstumsfaktor-haltige serumfreie Medium in Schritt e) bFGF und PDGF umfaßt. Ferner bevorzugt ist ein Verfahren in dem die gereinigte neurale Vorläuferzelle in einem Injektionsmedium aufgenommen wird.

Die Erfindung betrifft ferner ein Verfahren zur Herstellung der gereinigten Vorläuferzellen mit neuronalen oder glialen Eigenschaften, umfassend die folgenden Schritte: a') proliferieren von ES-Zellen, b') kultivieren der ES-Zellen aus Schritt a') zu neuralen Vorläuferzellen, c') proliferieren der neuralen Vorläuferzellen in einem Wachstumsfaktor-haltigen serumfreien Medium, d') proliferieren der Vorläuferzellen aus Schritt c') in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium zu neuralen Sphäroiden und isolieren der neuralen Sphäroide und e') proliferieren der neuralen Sphäroide aus Schritt d') in einem Wachstumsfaktor-haltigen serumfreien Medium bis zur Ausbildung eines aus glialen Vorläuferzellen bestehenden Zellrasens und isolieren der gereinigten Vorläuferzellen. Bevorzugt ist eine Proliferation der ES-Zellen in Schritt a') zu Zellaggregaten, insbesondere Embryoid Bodies. Bevorzugt ist ferner ein Verfahren f'), wobei die in Schritt e') gewonnenen glialen Vorläuferzellen durch die Zugabe einzelner Faktoren in eine astrozytäre oder oligodendrogliale Richtung gesteuert und isoliert werden. Besonders bevorzugt ist ein Verfahren, wobei das Wachstumsfaktor-haltige serumfreie Medium in Schritt c') bFGF umfaßt. Ferner besonders bevorzugt ist ein Verfahren, wobei das Wachstumsfaktor-haltige serumfreie Medium in den Schritten d'), e') und f') die Wachstumsfaktoren bFGF und EGF einzeln oder in Kombination umfaßt. Ferner besonders bevorzugt ist ein Verfahren, wobei in Schritt f') für die Induktion einer astrozytären Differenzierung der "ciliary neurotrophic factor" (CNTF) und für die Induktion und Ausreifung oligodendroglialer Zellen das Schilddrüsenhormon T3 benützt werden. Ferner bevorzugt ist ein Verfahren in dem die gereinigte neurale Vorläuferzelle in einem Injektionsmedium aufgenommen wird.

Die erfindungsgemäßen neuralen Vorläuferzellen können als Arbeitsmittel (Arzneimittel) in der Medizin verwendet werden. Bevorzugt ist die Verwendung der gereinigten neuralen Vorläuferzellen zur Herstellung eines Arbeitsmittels (Arzneimittels) zur Therapie von neuralen Defekten. Besonders bevorzugt ist die Verwendung der gereinigten neuralen Vorläuferzellen zur Rekonstitution von neuronalen Zellen oder zur Remyelinisierung demyelinisierter Nervenzellen insbesondere demyelinisierter Bereiche des Nervensystems mittels Zelltransplantation ins Nervensystem.

Insbesondere bevorzugt ist die Rekonstitution neuronaler Zellen, welche im Rahmen traumatischer, ischämischer, degenerativer, genetischer (anlagebedingter), hypoxischer, metabolischer, infektiöser, neoplastischer oder toxischer Schädigungen des Nervensystems in ihrer Funktion gestört oder ausgefallen sind. Bevorzugte Schädigungen des Nervensystems, auf die dieses Verfahren angewandt werden kann, umfassen unter anderen traumatische Hirn- und Rückenmarksverletzungen, ischämische und hämorrhagische Infarkte von Abschnitten des Nervensystems, M. Parkinson, M. Huntington, M. Alzheimer, anlagebedingte Atrophien insbesondere des Kleinhirns und des Hirnstammes, Motoneuronerkrankungen und spinale Formen der Muskelatrophie. Weiter bevorzugt ist die Rekonstitution neuronaler Zellen, welche im Rahmen des physiologischen Alterungsprozesses in ihrer Funktion gestört oder ausgefallen sind. Ferner insbesondere bevorzugt ist die Remyelinisierung demyelinisierter Hirnareale. Bevorzugte Schädigungen des Nervensystems, auf die die Transplantation der verfahrensgemäß gewonnenen glialen Vorläuferzellen als remyelinisierende Maßnahme angewandt werden können, umfassen unter anderen die multiple Sklerose (MS), die Adrenoleukodystrophie sowie die Pelizaeus-Merzbacher'sche Erkrankung.

Ferner bevorzugt ist die Verwendung der neuralen Vorläuferzellen für zellvermittelten Gentransfer in das Nervensystem. Bevorzugte Schädigungen des Nervensystems, auf die die Transplantation der verfahrensgemäß gewonnenen glialen Vorläuferzellen in Form eines zellvermittelten Gentransfers anwendbar sind, umfassen unter anderen insbesondere auf angeborenen Enzymdefekten beruhende metabolische Erkrankungen sowie Neoplasien innerhalb des Nervensystems. Die erfindungsgemäßen Vorläuferzellen können ferner für die *in vitro* Produktion von klinisch und gewerblich nutzbaren Faktoren z.B. Polypeptiden benützt werden.

Die Erfindung wird durch Abbildungen weiter erläutert.

Abbildung 1 ist eine schematische Darstellung (ein Fließschema) der Gewinnung neuraler Vorläuferzellen aus ES-Zellen. A zeigt ES-Zellen (leere Kreise) auf einem Feeder-Zellrasen embryonaler Fibroblasten (kleine Rechtecke). Im Anschluß an eine Proliferation können die ES-Zellen weiterverarbeitet oder in flüssigem Stickstoff tiefgefroren werden. B zeigt ES-Zellen in Gelatine-beschichteten Feederzell-freien Zellkulturschalen. C zeigt Embryoid Bodies, in

denen eine beginnende Differenzierung u.a. in neurale Zellen (schwarze Kreise) stattfindet. D zeigt plattierte Embryoid Bodies in einer Zellkulturschale, die ITSFn Medium enthält. Dieses serumfreie Medium begünstigt das Überleben neuraler Zellen (schwarze Kreise). E zeigt proliferierte neurale Vorläuferzellen, die in einem Wachstumsfaktor-haltigen serumfreien Medium (sog. N3FL Medium) in Anwesenheit von bFGF weiter proliferieren. F zeigt proliferierte neurale Vorläuferzellen, die in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium (sog. N3EFL Medium) in Anwesenheit von bFGF und EGF weiter proliferieren. G zeigt proliferierte neurale Vorläuferzellen, die in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium (sog. N2FP Medium) in Anwesenheit von bFGF und PDGF weiter proliferieren. Nach 2 Passagen in N2FP Medium können die Zellen für remyelinisierende Transplantate verwendet oder für eine spätere Verwendung in serumfreiem Gefriermedium tiefgefroren werden.

Abbildung 2 ist eine schematische Darstellung (ein Fließschema) der Gewinnung neuraler Sphäroide und der daraus abgeleiteten Zellpopulationen aus ES-Zellen. A bis E sind identisch mit Abbildung 1. F zeigt aus N3FL Kulturen in unbeschichteten Zellkulturschalen etablierte neurale Sphäroide, die in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium (sog. N2EF Medium) in Anwesenheit von bFGF und EGF proliferieren und auch kryokonserviert werden können. G zeigt die *in vitro* Differenzierung solcher Sphäroide durch Wachstumsfaktorentzug, wobei reife Neurone, Astrozyten und Oligodendrozyten entstehen. H zeigt die Transplantation solcher Sphäroide in das Nervensystem. I zeigt die Entstehung glialer Vorläuferzellpopulationen in Form sog. Touch-down Kulturen. Hierbei werden die in F erhaltenen Sphäroide in Anwesenheit von Wachstumsfaktoren solange gezüchtet, bis sie beginnen, sich an unbeschichtete Zellkulturschalen anzuheften und gliale Vorläuferzellen aus den Sphäroiden auf die Zellkulturschale auswandern. K zeigt, wie nach Entfernen der adhärierten Sphäroide die auf die Zellkulturschale ausgewanderten Vorläuferzellen in Anwesenheit von Wachstumsfaktoren weiter proliferiert werden. Die in I und K erzeugten Zellen können kryokonserviert werden. Ferner ist es möglich, die in K gewonnenen glialen Vorläuferzellen ins Nervensystem zu transplantieren. L und M zeigen, wie durch Zugabe von CNTF oder T3 und konsekutivem Wachstumsfaktorentzug eine astrozytäre (L) oder

oligodendrogliale (M) Differenzierung der aus ES Zellen gewonnenen glialen Vorläuferzellen induziert werden kann.

Abbildung 3 zeigt Zellkulturen von neuralen Vorläuferzellen nach *in vitro* Proliferation und Differenzierung von ES-Zellen. A und B zeigt von aus J1-ES-Zellen abgeleitete neurale Vorläuferzellen während der Proliferation in N2FP Medium in Phasenkontrast-Aufnahmen. C und D zeigt eine oligodendrogliale Differenzierung von N2FP Kulturen 4 Tage nach Entzug der Wachstumsfaktoren bFGF und PDGF. Zahlreiche Zellen exprimieren das oligodendrogliale Markerantigen O4. C ist eine Immunfluoreszenzaufnahme, D ist die korrespondierende Phasenkontrastaufnahme. E und F zeigt eine astrozytäre Differenzierung von N2FP Kulturen. Vier Tage nach Wachstumsfaktorentzug lassen sich zahlreiche GFAP-positive Astrozyten nachweisen. E ist eine Immunfluoreszenzaufnahme, F ist die korrespondierende Phasenkontrastaufnahme. G und H zeigt eine neuronale Differenzierung in N2FP Kulturen. Zusätzlich zu oligodendroglialen und astrozytären Zellen lassen sich nach Wachstumsfaktorentzug Neurone nachweisen. Diese weisen lange Fortsätze auf und zeigen eine deutliche Expression des neuronalen Markerantigens β-III-Tubulin. G ist eine Immunfluoreszenzaufnahme, H ist die korrespondierende Phasenkontrastaufnahme.

Abbildung 4 zeigt Vibratomschnitte eines Empfängergehirns nach Transplantation von aus ES-Zellen gewonnenen glialen Vorläuferzellen. Die glialen Vorläuferzellen differenzieren nach Transplantation in fetales Rattengehirn in Oligo- und Astroglia aus. Die aus ES-Zellen hergestellten Oligodendrozyten myelinisieren das Empfängergehirn. (A) zeigt von den transplantierten Zellen abstammende Astrozyten, die nach intraventrikulärer Transplantation in ein fetales Rattengehirn in das Hirnparenchym einwandern. Für dieses Experiment wurden N2FP Kulturen als Einzelzellsuspension in das Ventrikelsystem 17 Tage alter Rattenembryonen injiziert. Die Empfängertiere wurden drei Wochen nach der Geburt analysiert. Für den Nachweis der Donorzellen wurde ein Antikörper gegen das Maus-spezifische gliale Antigen M2 verwendet. Am oberen Bildrand ist der III. Ventrikel zu erkennen. (B) zeigt Inkorporation von aus ES-Zellen abgeleiteter Glia in die Großhirnrinde einer neugeborenen Ratte. Die Zellen waren am Embryonaltag 16 in das Ventrikelsystem des Empfängers implantiert worden. Die Darstellung erfolgte über eine Immunfluoreszenzuntersuchung mit einem Antikörper gegen das Maus-spezifische Antigen M6. Die Zellen weisen eine charakteristische astrozytäre Morphologie auf. (C) zeigt Inkorporation von aus ES-Zellen abgeleiteten Oligodendrozyten in

die unteren Vierhügel (*colliculus inferior*) einer 22 Tage alten myelindefizienten Ratte, welche am Embryonaltag 17 eine intraventrikuläre Injektion einer N2FP Kultur erhalten hatte. Die Donorzellen sind mittels einer DNA *in situ* Hybridisierung mit einem DNA-Sondenmolekül gegen Maus-Satelliten-DNA identifiziert worden (schwarz gefärbte Zellkerne). Die inkorporierten Zellen haben begonnen, das Empfängergehirn zu myelinisieren. Da myelindefiziente Ratten kein immunreaktives PLP bilden, konnte die Myelinisierung immunhistochemisch mit einem Antikörper gegen PLP nachgewiesen werden (schwarze Fortsätze). (D) zeigt eine Nahaufnahme eines aus ES-Zellen abgeleiteten myelinisierenden Oligodendrozyten nach Inkorporation in den Hypothalamus eines 22 Tage alten Empfängertieres. Man erkennt deutlich den hybridisierten Kern und die PLP-positiven Fortsätze in typischer paralleler Anordnung.

Abbildung 5 zeigt Kulturen von aus ES Zellen gewonnenen neuralen Sphäroiden und aus ihnen gewonnene Zellpopulationen. A zeigt 5 Tage alte, aus der ES Zellinie J1 gewonnene neurale Sphäroide wie sie z.B. für die Transplantation ins Nervensystem verwendet werden können. B zeigt zwei Wochen alte, aus J1-ES-Zellen gewonnene neurale Sphäroide, gefärbt mit einem Antikörper gegen das typischerweise in neuralen Vorläuferzellen exprimierte Intermediärfilament Nestin. Zu diesem Zeitpunkt haben die Sphäroide eine makroskopisch sichtbare Größe erreicht (Immunfluoreszenzaufnahme). C und D zeigen 5 Tage alte, aus der ES Zellinie CJ7 gewonnene neurale Sphäroide, welche in mit Polyornithin und Fibronektin beschichteten Zellkulturschalen ausgesät und für weitere 5 Tage ohne Wachstumsfaktoren kultiviert worden waren. Unter diesen Bedingungen desintegrieren die Sphäroide und differenzieren in neurale Zellen aus. Gezeigt ist in D eine Doppelimmunfluoreszenz-Untersuchung mit Antikörpern gegen das neuronale Antigen β-III-Tubulin (helleres Signal) und das für neurale Vorläuferzellen typische Intermediärfilament Nestin (dunkleres Signal, Pfeile). Der Vergleich mit der korrespondierenden Phasenkontrast-Aufnahme (C) läßt erkennen, daß jede der gezeigten Zellen zumindest einen dieser neuralen Marker exprimiert. E und F zeigt eine für 7 Tage auf Polyornithin und Fibronektin in Abwesenheit von Wachstumsfaktoren differenzierte, aus der ES Zellinie J1 gewonnene 5 Tage alte neurale Sphäroid-Kultur, gefärbt mit Antikörpern gegen den neuronalen Marker Microtubule-Associated Protein 2 (MAP2; E) und den Neurotransmitter GABA (F) (Immunfluoreszenz-Aufnahmen). Es ist erkenntlich, daß zahlreiche Neurone den Neurotransmitter GABA exprimieren. Zellpopulationen, welche reich an GABAergen Zellen sind, können für die

Transplantation von M. Huntington-Patienten verwendet werden. G und H zeigen Zellen derselben Kultur, gefärbt mit Antikörpern gegen den neuronalen Marker Microtubule-Associated Protein 2 (MAP2; G) und den Neurotransmitter Glutamat (Immunfluoreszenz-Aufnahmen). Es ist erkenntlich, daß zahlreiche Neurone den Neurotransmitter Glutamat exprimieren. I zeigt eine für zwei Wochen auf Polyornithin und Fibronektin in Abwesenheit von Wachstumsfaktoren differenzierte, aus der ES Zellinie J1 gewonnene 5 Tage alte neurale Sphäroid-Kultur, doppelgefärbt mit Antikörpern gegen die neuronalen Antigene β-III-Tubulin (filamentäre Färbung in Zellfortsätzen) und Synapsin (punktförmiges Signal, den Fortsätzen anhaftend). Um die Ausreifung der Neurone zu unterstützen, wurde während der letzten 5 Tage der Differenzierung das Neurotrophin Brain-Derived Neurotrophic Factor (BDNF) zugegeben (20 ng/ml). Es ist erkenntlich, daß die aus den neuralen Sphäroiden gewonnenen Neurone sehr reife Morphologien ausbilden und eine Expression von für die neuronale Signalübertragung wichtigen synaptischen Proteinen aufweisen. K zeigt eine auf Polyornithin und Fibronektin in Abwesenheit von Wachstumsfaktoren differenzierte, aus der ES Zellinie J1 gewonnene 5 Tage alte neurale Sphäroid-Kultur, gefärbt mit einem Antikörper gegen Tyrosinhydroxylase. Tyrosinhydroxylase-positive Neurone werden z.B. für die Transplantation von Parkinson-Patienten verwendet. Diese Immunfluoreszenz-Aufnahme verdeutlicht, daß zahlreiche der aus den ES Zellen gewonnenen Neurone dieses Enzym bilden. L zeigt ein für eine Woche auf Polyornithin und Fibronektin in Abwesenheit von Wachstumsfaktoren differenziertes, aus der ES Zellinie J1 gewonnenes 11 Tage altes neurales Sphäroid, gefärbt mit einem Antikörper gegen das astrozytäre Antigen Glial Fibrillary Acidic Protein (GFAP). Diese Immunfluoreszenz-Aufnahme verdeutlicht, daß die aus ES Zellen hergestellten neuralen Sphäroide auch Astrozyten generieren. M zeigt eine für 5 Tage auf Polyornithin und Fibronektin in Abwesenheit von Wachstumsfaktoren differenzierte, aus der ES Zellinie J1 gewonnene 5 Tage alte neurale Sphäroid-Kultur, gefärbt mit einem Antikörper gegen den oligodendroglialen Marker O4. Diese Immunfluoreszenz-Aufnahme verdeutlicht, daß die aus ES Zellen hergestellten neuralen Sphäroide auch Oligodendrozyten generieren.

Abbildung 6 zeigt aus neuralen Sphäroiden gewonnene gliale Vorläuferzellen. A zeigt ein typisches Beispiel einer in Entstehung begriffenen sog. Touch-down Kultur. Aus der ES-Zellinie J1 gewonnene neurale Sphäroide adhärieren an unbeschichtete Zellkulturschalen, und es kommt zum Auswandern zahlreicher glialer Zellen aus den Sphäroiden. Die adhärierten Sphäroide lassen sich durch Schütteln leicht wieder von der Zellkulturschale lösen, wobei ein

reiner glialer Vorläuferzellrasen zurückbleibt. B zeigt eine Immunfluoreszenz-Untersuchung einer derartig aus der ES Zellinie J1 gewonnenen glialen Vorläuferzell-Population mit einem Antikörper gegen das neurale Antigen A2B5. Es ist erkenntlich, daß die gewonnenen glialen Vorläuferzellen dieses neurale Antigen exprimieren.

Abbildung 7 zeigt die Steuerung der glialen Differenzierung von aus ES Zellen gewonnenen glialen Vorläuferzellen durch Zugabe einzelner Faktoren. Hierbei wurde einer aus der ES Zellinie J1 gewonnenen sog. Touch-down Kultur, welche in Anwesenheit von EGF und FGF kultiviert worden war, zusätzlich die Faktoren CNTF (10 ng/ml) oder T3 (3 ng/ml) zugesetzt. 2 Tage später wurden die Wachstumsfaktoren entzogen, CNTF und T3 jedoch weiter täglich zugesetzt. Nach weiteren 5 Tagen wurden die Kulturen fixiert und mit Antikörpern gegen das astrozytäre Antigen GFAP und das oligodendrogliale Antigen O4 gefärbt. Die Abbildung, welche jeweils Immunfluoreszenz- und korrespondierende Phasenkontrast-Aufnahmen zeigt, verdeutlicht, daß die Zugabe von CNTF oder T3 zu den aus ES Zellen gewonnenen Vorläuferzellen die Differenzierung derselben beinflußt. In Anwesenheit von CNTF bildet der Großteil der Zellen eine astrozytäre Morphologie aus und exprimiert den astrozytären Marker GFAP. In Anwesenheit von T3 hingegen finden sich zahlreiche Zellen mit sternförmiger, oligodendroglialer Morphologie und Expression des oligodendroglialen Makers O4.

Abbildung 8 zeigt die neuronale Differenzierung von aus der ES Zellinie J1 gewonnenen neuralen Sphäroiden *in vitro* (A und B) und nach Transplantation in das Nervensystem einer Ibotensäure-behandelten Ratte (ein Tiermodell für die Huntington'sche Erkrankung, C und D). Hierbei wurden 5 Tage alte neurale Sphäroide in das Striatum einer adulten, Ibotensäure-behandelten Ratte implantiert. Ein Teil der Sphäroide wurde parallel *in vitro* durch Wachstumsfaktorentzug für 5 Tage differenziert und dann mit einem Antikörper gegen den Neurotransmitter GABA gefärbt (A, Immunfluoreszenz-Aufnahme; B, korrespondierende Phasenkontrast-Aufnahme). Es ist erkenntlich, daß zahlreiche der aus ES Zellen gewonnenen Nervenzellen den Neurotransmitter GABA exprimieren. 7 Wochen nach Implantation in das adulte Rattengehirn läßt sich ein vitales Transplantat mit neuronaler Differenzierung nachweisen (C und D). C zeigt eine Übersichtsaufnahme eines Transplantats, welches mit einem Antikörper gegen das Maus-spezifische neurale Antigen M6 gefärbt wurde (Immunfluoreszenz). Man erkennt ein homogen gefärbtes, in das Empfängergehirn inkorporiertes Transplantat. Bei diesem Tier war auch eine funktionelle Verbesserung

aufgetreten. Das durch die Ibotensäure-Läsion hervorgerufene Rotationsverhalten konnte normalisiert werden. D zeigt von einem solchen Transplantat ausgehende, in das Empfängergehirn einsprossende Axone. Auch hierbei handelt es sich um eine Immunfluoreszenz-Untersuchung mit einem Antikörper gegen M6.

Als Ausgangsmaterial für die erfindungsgemäßen neuralen Vorläuferzellen können embryonale Stammzellen, z.B. der Maus, zunächst auf einem Rasen nicht teilungsfähiger embryonaler Fibroblasten in serumhaltigem Medium nach üblichen Verfahren (Hogan et al., Manipulating the Mouse Embryo, Cold Spring Harbor Press, New York, 1994) zu einer gewünschten Zellmenge proliferiert werden. Neben etablierten ES-Zellinien, z.B. die embryonalen Maus-Stammzellinien J1 (Li et al., Cell 69:915-926, 1992), R1 (Nagy et al. Proc. Natl. Acad. Sci. USA 90:8424-8428, 1993) und CJ7 (Swiatek & Gridley, Genes Dev. 7:2071-2084, 1993) können auch ES-Zellen aus Embryonen, beispielsweise aus 3 bis 4 Tage alten Blastozysten der Maus, gewonnen werden (Hogan et al., Manipulating the Mouse Embryo, Cold Spring Harbor Press, New York, 1994). Ferner können ES-Zellen auch aus anderen Spezies verwendet werden. Typische Beispiele isolierter ES-Zellen umfassen Ratte (Iannaconne et al., Dev. Biol. 163:288-292, 1994), Hamster (Doetschman et al., Dev. Biol. 127:224-227, 1988), Vogel (Pain et al., Development 122:2339-2348, 1996), Fisch (Sun et al., Mol. Mar. Biol. Biotechno. 4:193-199, 1995), Schwein (Wheeler, Reprod. Fertil. Dev. 6:563-568, 1994), Rind (First et al., Reprod. Fertil. Dev. 6:553-562), Primaten (Thomson et al., Proc. Natl. Acad. Sci. USA 92:7844-7848, 1995) oder ES-Zellen aus menschlichem Embryonalgewebe. Mehrere Monate nach Einreichung der prioritätsbegründenden deutschen Patentanmeldung Nr. 197 56 864.5 gelang es zwei Forschergruppen, ES Zellen bzw. ES-Zell-ähnliche Stammzellen aus menschlichem Embryonalgewebe zu isolieren (Thomson et al., Science 282: 1145-1147, 1998; Shamblott et al., Proc. Natl. Acad. Sci. USA 95: 13726-13731, 1998).

Neuere Studien haben zudem gezeigt, daß Embryonen und damit auch embryonale Stammzellen durch Transplantation von Zellkernen aus Zellen eines ausgereiften Organismus in unbefruchtete Eizellen erhalten werden können (Wilmut et al., Nature 385:810-813, 1997). Für den Fachmann ist ersichtlich, daß eine Kombination der Kerntransplantation mit dem erfindungsgemäßen Verfahren die Herstellung autologer neuraler Vorläuferzellen aus differenzierten Zellen desselben Organismus erlaubt. Da die Erzeugung von Embryonen durch Transplantation von aus differenzierten Zellen gewonnenen Zellkernen in unbefruchtete

Eizellen bereits an großen Säugetieren wie dem Schaf gezeigt wurde (Wilmut et al., Nature 385:810-813, 1997), ist dieses Verfahren auch bei menschlichen Zellen durchführbar. Ferner können ES-Zellen oder ES-Zell-ähnliche Zellen aus embryonalen Keimzellen (*embryonic germ cells*) erhalten werden. Die nach dem für diese Patentanmeldung geltenden Prioritätsdatum erschienenen Arbeiten über die Isolierung von ES-Zellen aus menschlichen Keimbläschen (Blastozysten; Thomson et al., Science 282: 1145-1147, 1998) bzw. von ES-Zell-ähnlichen Stammzellen aus humanen embryonalen Keimzellen (Shamblott et al., Proc. Natl. Acad. Sci. USA 95: 13726-13731, 1998) weisen darauf hin, daß diese Verfahren auf den Menschen übertragbar sind.

Von Feeder-Zellen abhängige ES-Zellen können dann z.B. nach dem von Okabe et al. beschriebenen Verfahren in Gelatine-beschichteten Schalen ausgesät und anschließend in unbeschichteten Petrischalen in der Abwesenheit von LIF zu Embryoid Bodies aggregiert werden (Okabe et al., Mech. Dev. 59:89-102, 1996). Drei bis vier Tage alte Embryoid Bodies können dann auf Zellkulturschalen ausplattiert und für 4-8 Tage in einem serumfreien Medium (ITSFn Medium) gezüchtet werden (Okabe et al., Mech. Dev. 59:89-102, 1996). Während dieser Zeit kommt es zu massivem Zelltod unter nicht neuralen Zellen.

Nach 4-8 Tagen in ITSFn Medium können die Zellen in eine Einzelzellsuspension trituriert (wiederholtes Aufziehen und Ablassen von Flüssigkeit mittels einer enghalsigen Pipette) und in N3FL Medium überführt werden. Es handelt sich hierbei um ein serumfreies Medium, welchem bFGF zugesetzt wird (Okabe et al., Mech. Dev. 59:89-102, 1996). bFGF hat einen proliferativen Effekt auf neurale Vorläuferzellen.

Nach 4-8 Tagen in N3FL Medium beginnt die eigentliche Bereitstellung glialer und neuronaler Vorläuferzellpopulationen in Form eines Zellrasens. Im Gegensatz zu neuronalen Zellen zeichnen sich Gliazellen und insbesondere ihre unmittelbaren Vorläufer durch eine starke Proliferationsfähigkeit aus. Das erfindungsgemäße Verfahren nützt diese Eigenschaft aus. Die vorstehend beschriebenen N3FL-Kulturen werden nacheinander in verschiedene serumfreie, Wachstumsfaktor-haltige Medien überführt. Während dieser Phase kommt es zu einer sehr starken Anreicherung bipotenter oligo-astrozytärer Vorläuferzellen bei gleichzeitiger Differenzierung und Elimination primitiver embryonaler und nicht-neural differenzierter Zellen.

Hierzu werden die in N3FL Medium kultivierten Zellen in üblicher Weise mechanisch von der Kulturschale gelöst, in eine Einzelzellsuspension trituriert und in serumfreies Medium überführt, welches z.B. die Wachstumsfaktoren bFGF und EGF (N3EFL Medium) enthält. In diesem Medium werden die Zellen bis zur Subkonfluenz als Zellrasen propagiert.

Die erhaltenen Zellen können nach etwa zwei Passagen in diesem Medium für Transplantationszwecke verwendet werden. Für eine weitergehende Aufreinigung kann jedoch ein zusätzlicher Schritt durchgeführt werden.

Die in N3EFL Medium kultivierten Zellen können auch erneut in üblicher Weise von der Kulturschale abgelöst, in eine Einzelzellsuspension trituriert, und erneut in einem serumfreien Medium ausplattiert werden, das eine zweite Wachstumsfaktorkombination z.B. bFGF und PDGF enthält.

Die so erhaltenen gereinigten neuralen Vorläuferzellen können weiteren Passagen in diesem Medium unterworfen werden. Nach etwa zwei Passagen besteht die Population aus gereinigten neuralen Vorläuferzellen, die direkt oder vorzugsweise nach Isolierung für z.B. remyelinisierende Transplantate verwendet werden können. Mikroskopisch erkennt man eine homogene Population bipolarer bis sternförmiger Zellen, welche die Kulturschale in Form eines gleichmäßig verteilten Zellrasens ausfüllen. Zu diesem, aber auch bereits zu einem früheren Zeitpunkt kann die neurale Vorläuferzellpopulation in serumfreiem Gefriermedium gefroren und zu einem späteren Zeitpunkt in üblicher Weise wieder aufgetaut werden, ohne ihre Vorläuferzelleigenschaften zu verlieren. Werden die Wachstumsfaktoren für mehrere Tage nicht zugesetzt, d.h. entzogen, kommt es zu einer beginnenden Differenzierung *in vitro*. Immunhistochemisch lassen sich dann neben Markerantigenen für neurale Vorläuferzellen (z.B. Nestin), auch oligodendrogliale (z.B. O4) und astrozytäre (z.B. GFAP) Antigene nachweisen. O4-positive Zellen können dabei bereits 30 - 70% und GFAP-positive Zellen 20 - 40% der Zellpopulation ausmachen. Darüber hinaus finden sich unter diesen Bedingungen Zellen, welche neuronale Antigene exprimieren.

Nach 4-8 Tagen in N3FL Medium beginnt ebenfalls die eigentliche Bereitstellung glialer und neuronaler Vorläuferzellpopulationen in Form von neuralen Sphäroiden.

Zur Anreicherung neuraler Zellen werden die in N3FL Medium kultivierten Zellen in üblicher Weise mechanisch von der Kulturschale gelöst, in eine Einzelzellsuspension trituriert und diese in unbeschichteten Zellkulturschalen in serumfreiem Medium kultiviert, welches z.B. die Wachstumsfaktoren bFGF und EGF enthält. Dabei entstehen innerhalb weniger Tage sphäroide Zellaggregate (neurale Sphäroide), welche zum überwiegenden Teil aus Nestin-positiven neuralen Vorläuferzellen bestehen. Die neuralen Sphäroide können freischwimmend im Zellkulturüberstand propagiert werden. Differenzierte neurale und nicht neurale Zellen zeigen hingegen eine starke Tendenz, sich an die Oberfläche der Zellkulturschalen anzuheften.

Sobald sich kleine Sphäroide gebildet haben, werden diese der Kultur entnommen und in neue unbeschichtete Zellkulturschalen überführt. Solche Sphäroide können bereits nach wenigen (5-7) Tagen für Transplantationszwecke verwendet werden. Hierbei differenzieren die in den Sphäroiden enthaltenen neuralen Vorläuferzellen in reife neurale Zellen aus, welche das Empfängergehirn innervieren. Undifferenzierte kleine Sphäroide können in serumfreiem Gefriermedium in flüssigem Stickstoff gefroren werden und zu einem späteren Zeitpunkt aufgetaut und differenziert werden.

Ein weiterer Gegenstand der Erfindung sind differenzierte neurale Zellen in Form von Sphäroiden, die sich zur Transplantation eignen. Sie können durch *in vitro*-Induktion der Differenzierung von erfindungsgemäßen Vorläuferzellen mit neuronalen oder glialen Eigenschaften hergestellt werden. Hierzu werden die Wachstumsfaktoren entzogen und die Sphäroide mit den undifferenzierten neuralen Vorläuferzellen in z.B. mit Polyornithin und Fibronektin beschichtete Zellkulturschalen ausgesät. Die Sphäroide heften sich dann rasch an die Oberfläche der Zellkulturschale an und bilden neben Nestin-positiven neuralen Vorläuferzellen Neurone, Astrozyten und Oligodendrozyten. Die anhaftenden Nervenzellen können weiter ausgereift werden und exprimieren dann eine Vielzahl neuronaler Marker, beispielsweise MAP2, β-III-Tubulin, Synapsin, Cholinacetyltransferase, Tyrosinhydroxylase, GABA, Glutamat, Serotonin, Peripherin und Calbindin. Die Ausreifung und das Überleben der ausgereiften Neurone kann durch Zugabe von Neurotrophinen, z.B. Brain-Derived Neurotrophic Factor (BDNF) oder Neurotrophin-3 (NT-3) zum Zellkulturmedium begünstigt werden.

Die Sphäroide können bis zu mehreren Wochen in serumfreiem Medium, welches z.B. die Wachstumsfaktoren bFGF und EGF enthält, in Suspensionskultur gehalten werden. Die Sphäroide können dabei soweit an Größe zunehmen, daß sie auch makroskopisch leicht zu erkennen sind. Innerhalb der Sphäroide kommt es unter solchen Bedingungen zu einer zunehmenden Zelldifferenzierung. Eine derartige Differenzierung von ES-Zellen in freischwimmenden Sphäroiden macht es physikalisch möglich, aus ES-Zellen gewonnene Neurone auch in differenzierterem Zustand zu transplantieren, z.B. in Form solcher Sphäroide. Dies ist nach Differenzierung von einer Kulturschale anhaftenden Neuronen nicht möglich, da die zahlreichen der Oberfläche anhaftenden Fortsätze beim Ablösen in der Regel beschädigt und die Neurone dadurch zerstört werden.

Während der letzten Jahre sind zahlreiche Faktoren identifiziert worden, die in der Lage sind, auf die Differenzierung von Nervenzellverbänden einzuwirken. Solche Faktoren können beispielsweise innerhalb von Nervengewebe eine Polarisierung herbeiführen. So wurde beispielsweise gezeigt, daß das Produkt des Gens *Sonic hedgehog* in Nervengewebe einen ventralen Phänotyp induzieren kann (Ericson et al., Cell 81:747-756, 1995). Es ist zu erwarten, daß derartige Faktoren auch an künstlich aus ES-Zellen hergestellten neuralen Zellverbänden wirksam sind. Für den Fachmann ist ersichtlich, daß die Applikation derartiger Phänotyp-bestimmender Faktoren es erlauben wird, Neurone und Glia von einem spezifischen Phänotyp gezielt herzustellen. Ein Beispiel könnte die gezielte Induktion von Nervenzellen von ventralem mesencephalem Phänotyp sein, wie sie für die Transplantation z.B. von Parkinson-Patienten benötigt werden. Eine derartige Induktion dopaminerger ventraler mesencephaler Neurone durch Sonic Hedghog wurde in Fragmenten natürlich gewachsenen Nervengewebes bereits nachgewiesen (Wang et al., Nature Med. 1:1184-1188, 1995).

Für die Herstellung glialer Vorläuferzellen aus von ES-Zellen erhaltenen neuralen Sphäroiden werden die Sphäroide solange in Wachstumsfaktor-haltigem serumfreiem Medium in Suspensionskultur gehalten, bis sie beginnen, sich leicht an die unbeschichtete Oberfläche der Zellkulturschale anzuheften. Dabei können als Wachstumsfaktoren z.B. bFGF und EGF (allein und in Kombination) verwendet werden. Nach Anheften der Sphäroide wandern Zellen von glialer Morphologie aus den Sphäroiden auf die Zellkulturplatten aus (sog. Touch-down Zellen). Die Entstehung dieser Zellpopulation ist ungeklärt. Es ist jedoch anzunehmen, daß im

Laufe der zunehmenden Zelldifferenzierung innerhalb der Sphäroide gliale Vorläuferzellen gebildet werden, die sich durch ein stärkeres Adhesions- und Migrationsverhalten auszeichnen. Solche Touch-down Zellen-produzierende Sphäroide können als Generatoren für gliale Vorläuferzellen benützt werden. Hierzu werden die Sphäroide für kurze Zeit (< 1 Tag) in unbeschichteten Zellkulturschalen kultiviert. Sobald sich gliale Zellen abgesetzt haben, werden die Sphäroide wieder mechanisch von der Schale gelöst und in eine neue Schale überführt. Zurück bleibt in solchen Fällen ein Ring glialer Vorläuferzellen, der weiter in Anwesenheit von Wachstumsfaktoren, beispielsweise bFGF und EGF, allein oder in Kombination, vermehrt werden kann.

Derartig gewonnene „Touch-down Zellen" zeigen eine Expression des neuralen Antigens A2B5 (Eisenbarth et al., Proc. Natl. Acad. Sci. USA 76:4913-4917, 1979) und differenzieren nach Wachstumsfaktorentzug in Astrozyten und Oligodendrozyten aus. Immunhistochemisch lassen sich dann Markerantigene für Oligodendrozyten (z.B. O4) und Astrozyten (z.B. GFAP) nachweisen. Undifferenzierte „Touch-down Zellen" können in serumfreiem Gefriermedium in flüssigem Stickstoff gefroren werden, ohne ihr Proliferations- und Differenzierungsvermögen zu verlieren. Derartig gewonnene gliale Vorläuferzellen können auch für Transplantationen ins Nervensystem verwendet werden

Die Differenzierung der aus den ES-Zellen gewonnenen glialen Vorläuferzellen kann durch Zugabe einzelner Faktoren beeinflußt werden. Zugabe von CNTF (ciliary neurotrophic factor) kurz vor und während des Wachstumsfaktor-Entzugs führt zu einer überwiegend astrozytären Differenzierung. Zugabe des Schilddrüsenhormons T3 während dieser Phase führt zu einer vermehrten Differenzierung in Oligodendrozyten. Die Zugabe von serumhaltigem Medium während oder nach Wachstumsfaktor-Behandlung führt zu einem starken Anstieg der Zahl astrozytärer Zellen in diesen Kulturen.

Dem Fachmann ist ersichtlich, daß die gefrorenen und wieder aufgetauten neuralen Vorläuferzellen ebenfalls für Transplantate verwendet werden können. Zu einem früheren Zeitpunkt eingefrorenen neuralen Vorläuferzellpopulationen werden nach dem in üblicher Weise durchgeführten Auftauen den entsprechenden Passagen unterzogen, um die homogene Population bipolarer bis sternförmiger Zellen zu erhalten.

Das erfindungsgemäße Verfahren kann ferner mit bekannten Zelltrennungs- und Sortierverfahren kombiniert werden. Beispielsweise können neurale Subpopulationen zu bestimmten Zeitpunkten des erfindungsgemäßen Verfahrens durch Immunfluoreszenz-gestützte Zellsortierung (FACS), Immunopanning oder ähnliche Verfahren abgetrennt werden. Eine detaillierte Sortierung und Subklassifizierung kann es ermöglichen, auf die Bedürfnisse des jeweiligen Patienten abgestimmte (und unter Umständen genetisch modifizierte) neurale Ersatzzellen *in vitro* zu erzeugen. Da sowohl ES-Zellen als auch die aus ihnen gewonnenen erfindungsgemäßen neuralen Vorläuferzellen ohne Verlust ihrer Eigenschaften tiefgefroren und wieder aufgetaut werden können, ist ein Aufbau entsprechender Zellbanken z.B. autologer Zellbanken möglich.

Die mit dem erfindungsgemäßen Verfahren erhältlichen neuralen Vorläuferzellen z.B. neuronale, oligodendrogliale und astrozytäre Zellen können in einer Menge und Reinheit hergestellt werden, wie sie z.B. für die Transplantation und Reparatur von Defekten im Nervensystem erforderlich sind. Die mit dem erfindungsgemäßen Verfahren erhältlichen erfindungsgemäßen neuralen Vorläuferzellen enthalten z.B. nur geringe bis keine Mengen von z.B. primitiven embryonalen und nicht-neuralen Zellen. Die Reinheit der erfindungsgemäßen neuralen Vorläuferzellen liegt weit über der Reinheit von etwa 85% wie sie mit dem Verfahren von Okabe et al. (Mech. Dev. 59:89-102, 1996) erreicht wurde. Mit dem erfindungsgemäßen Verfahren können neurale Vorläuferzellen mit einer Reinheit von bis zu 100% erhalten werden. Ferner können mit dem erfindungsgemäßen Verfahren große Mengen neurale Vorläuferzellen gewonnen werden, ohne wie bisher auf Hirngewebe zurückgreifen zu müssen. Die erfindungsgemäßen neuralen Vorläuferzellen können aus ES-Zellen, z.B. der Maus, Ratte, Hamster, Vogel, Fisch, Schwein, Rind, Primaten oder Mensch erhalten werden. Die ES-Zellen können als ES-Zellinie vorliegen oder aus Embryonen gewonnen werden. Ferner können die ES-Zellen durch Proliferation von Oocyten gewonnen werden. Die Oocyten können z.B. entkernt und mit einem Zellkern aus z.B. ausdifferenziertem Gewebe injiziert werden, so daß autologe Oocyten zur Gewinnung von ES-Zellen verwendet werden. ES-Zellen bzw. ES-Zell-ähnliche Zellen können auch aus embryonalen Keimzellen (*embryonic germ cells*) erhalten werden. Die ES-Zellen können ferner in üblicher Weise gentechnisch modifiziert werden. Z.B. kann durch homologe Rekombination ein defektes Gen durch ein "normales" Gen ersetzt

werden. Ferner können defekte Gene auf übliche Weise deletiert werden. Diese Verfahren sind z.B. in der Maus vielfach verwendet worden und sind Stand der Technik.

Da ES-Zellen sich rasch teilen und routinemäßig genetischen Modifikationen unterzogen werden, ist es auch möglich, große Mengen an genetisch modifizierten neuronalen und glialen Vorläuferzellen zu erhalten. In Verbindung mit dem ausgeprägten Wanderungsverhalten neuronaler und glialer Vorläuferzellen kann dies beispielsweise dafür benutzt werden, große Abschnitte des Nervensystems mit genetisch modifizierten Vorläuferzellen zu besiedeln, welche lokal fehlende Substanzen substituieren oder Polypeptide mit protektiver oder anderer Wirkung sezernieren. Die genetische Modifizierbarkeit der Zellen kann auch dazu benutzt werden, die Expression von Abstoßungsreaktionen hervorrufenden Oberflächenantigenen durch Elimination der entsprechenden Gene zu beseitigen, was einen breiten klinischen Einsatz von aus ES-Zellen abgeleiteten Vorläuferzellen ohne Immunsuppression erlaubt.

Die erfindungsgemäßen neuralen Vorläuferzellen können als medizinische Arbeitsmittel zur Therapie von neuralen Defekten verwendet werden. Ein typisches Beispiel zur Verwendung der erfindungsgemäßen neuralen Vorläuferzellen ist die Rekonstitution funktionell defizienter oder verlorengegangener Nervenzellen durch transplantierte neurale Vorläuferzellen.

Um beispielsweise verlorengegangene Neurone zu rekonstituieren und damit verbundene neurologische Defizite zu bessern, werden z.B. die erfindungsgemäßen Sphäroide nach z.B. 4-7 Tagen in Suspensionskultur in Gehirnregionen implantiert, welche einen Verlust von Nervenzellen aufweisen. In derartigen Transplantaten lassen sich z.B. 6 Wochen nach Operation ausgereifte Nervenzellen beobachten, welche das Empfängergehirn innervieren. Die von den transplantierten Neuronen ausgehenden, in das Hirngewebe des Empfängers einsprossenden Axone können z.B. mit Antikörpern gegen Donor-spezifische neurale Antigene identifiziert werden. Diese Rekonstitution neuronaler Zellen ist funktionell. Dies läßt sich beispielsweise durch Verhaltenstests an Ibotensäure-behandelten Ratten vor und nach Transplantation belegen. Hierbei werden durch stereotaktische Injektion des Neurotoxins Ibotensäure in das Striatum zunächst große Mengen striataler Neurone zerstört. Der resultierende Defekt hat Ähnlichkeit zu der bei Menschen auftretenden Huntington'schen Erkrankung, weshalb dieses System auch vielfach als Tiermodell für die Huntington'sche Erkrankung benützt wird. Nach einseitiger Ibotensäureläsion läßt sich in den operierten Tieren

aufgrund der eingetretenen Asymmetrie durch Injektion bestimmter Drogen, z.B. Amphetamin, ein Rotationsverhalten induzieren, welches auch quantifizierbar ist. Ein Nervenzell-reiches Transplantat ist imstande, das Rotationsverhalten zu normalisieren. Dabei scheint insbesondere die Zahl der im Transplantat vorhandenen GABAergen Nervenzellen von Bedeutung zu sein. Das Ibotensäure-Läsionsmodell der Ratte und die Erfassung der funktionellen Kapazität neuraler Transplantate über eine Analyse des Rotationsverhaltens der Tiere sind Stand der Technik (Björklund et al., aus: Functional Neural Transplantation, Seiten 157-195, Raven Press, New York, 1994). Werden die erfindungsgemäßen neuralen Sphäroide in das Gehirn Ibotensäure-behandelter Ratten implantiert, kommt es postoperativ zu einer deutlichen Besserung des abnormen, durch die Ibotensäure-Läsion verursachten Rotationsverhaltens der Empfänger. Transplantationen von z.B. neuralen Zellen ins menschliche Nervensystem sind heute bereits klinisch durchführbar (Olanow et al., TINS 19:102-109, 1996).

Die erfindungsgemäßen, entweder aus den neuralen Sphäroiden oder aus dem Zellrasen hergestellten glialen Vorläuferzellen können ebenfalls als medizinische Arbeitsmittel zur Therapie von neuralen Defekten verwendet werden. Ein typisches Beispiel zur Verwendung der erfindungsgemäßen glialen Vorläuferzellen ist die Remyelinisierung demyelinisierter Hirnabschnitte durch transplantierte neurale Vorläuferzellen. Da demyelinisierende Erkrankungen oftmals große Abschnitte des Zentralen Nervensystems (ZNS) miteinbeziehen, können die erfindungsgemäßen glialen Vorläuferzellen, welche sich durch ein ausgeprägtes Wanderungsverhalten auszeichnen, ins ZNS transplantiert werden. Dabei kann eine lokalisierte Injektion genügen, um große Abschnitte des ZNS mit diesen Zellen zu infiltrieren und eine großflächige Remyelinisierung zu erreichen. Ein typisches Beispiel einer möglicherweise derartig therapierbaren Erkrankung ist die multiple Sklerose (MS). Bei dieser Erkrankung, deren Pathogenese noch ungeklärt ist, kommt es typischerweise zu einer multifokalen Demyelinisierung verschiedenster Hirnabschnitte. Durch die Transplantation der erfindungsgemäßen neuralen Vorläuferzellen kann eine Remyelinisierung solcher Defekte bewirkt werden. Das ausgeprägte Wanderungsverhalten der erfindungsgemäßen neuralen Vorläuferzellen kann dabei derartig ausgenutzt werden, daß von einer oder wenigen Implantationsstellen aus zahlreiche demyelinisierte Areale erreicht und repariert werden können.

Um beispielsweise eine Myelinisierung myelin-defizienter Hirnabschnitte herbeizuführen, werden die noch unter Wachstumsfaktor-Behandlung stehenden aus Monolayern (Zellrasen) oder neuralen Sphäroiden gewonnenen erfindungsgemäßen Zellen z.B. trypsinfrei von den Kulturschalen abgelöst und zu einer Einzelzellsuspension trituriert. Werden diese Zellsuspensionen in myelindefiziente Gehirnregionen implantiert, lassen sich beispielsweise etwa drei Wochen nach Implantation von den implantierten Zellen abstammende Oligodendrozyten und Astrozyten nachweisen. Die Myelinisierung der Nervenzellfortsätze des Empfängers läßt sich durch eine von den Donorzellen ausgehende Einscheidung dieser Fortsätze nachweisen, wobei in diesen Einscheidungen für Myelin charakteristische Proteine immunhistochemisch nachgewiesen werden können z.B. basisches Myelinprotein (MBP) und Proteolipid Protein (PLP).

Die erfindungsgemäßen neuralen Vorläuferzellen können ferner für die *in vitro* Produktion von klinisch und gewerblich nutzbaren Faktoren z.B. Polypeptiden benützt werden.

Die folgenden Beispiele erläutern die Erfindung und sind nicht als einschränkend zu verstehen.

Beispiel 1:

1.1. ES-Zell Proliferation

J1-ES-Zellen (Li et al., Cell 69:915-926, 1992) wurden zunächst auf einem Rasen von mitotisch inaktivierten embryonalen Fibroblasten in einem DMEM Medium (Life Technologies Nr. 11965) mit 20% fötalem Kälberserum (FBS, Life Technologies Nr. 10119) in Anwesenheit von 1.000 U/ml LIF (Life Technologies Nr. 13275) nach Standardmethoden (Hogan et al., Manipulating the Mouse Embryo, Cold Spring Harbor Press, New York, 1994) proliferiert. Neben FBS und LIF enthielt dieses Medium Standardkonzentrationen (Okabe et al., Mech. Dev. 59:89-102, 1996) nicht essentieller Aminosäuren (Life Technologies Nr. 11140) und der Nukleoside Adenosin (Sigma Nr. A-4036), Guanosin (Sigma Nr. G-6264), Cytidin (Sigma Nr. C-4654), Uridin (Sigma Nr. U-3003) und Thymidin (Sigma Nr. T-1895) sowie 0.1 mM 2-Mercaptoethanol (Sigma Nr. M-7522), 10 mM L-Glutamin (Sigma Nr. G-5763) und 25 mM HEPES (Sigma Nr. H-0763). In diesem wie in allen folgenden Schritten wurden die Zellen bei 37°C unter 5% CO_2 bei gesättigter Luftfeuchtigkeit (>85%) kultiviert.

1.2. Entfernung der Feeder Zellen

Sobald die ES-Zellen subkonfluent geworden waren, wurde die Kultur einmal mit 0,04% EDTA in PBS-Puffer gespült und die Zellen durch Zugabe einer Lösung von 0,05% Trypsin (Life Technologies Nr. 35400) und 0,04% EDTA in PBS abgelöst. Die Zellen wurden dann mehrfach durch eine Pasteurpipette zu einer Einzelzellsuspension trituriert und das Trypsin durch Zugabe serumhaltigen Mediums neutralisiert. Hierbei wurde der Trypsinlösung eine äquivalente Menge des bislang verwendeten Mediums zugesetzt. Nach Zentrifugation (5 min, 300 x g, RT) wurden die Zellen in dem bisher verwendeten Medium auf mit 0,2% Gelatine (Sigma Nr. G-2500) beschichtete 6 cm Zellkulturschalen ausplatiert (ca. 6 x 10^6 Zellen pro 6 cm Schale) und unter Anwesenheit von LIF (1.000 U / ml) weiter gezüchtet.

1.3. Herstellung von Embryoid Bodies

Sobald die Zellen erneut subkonfluent geworden waren (etwa nach 2-3 Tagen), wurden sie durch Zugabe einer Lösung von 0,05% Trypsin und 0,04% EDTA in PBS (ca. 1,5 ml pro 6 cm Schale) von der Gelatine abgelöst. Nach Ablösen der ES-Zell-Kolonien wurde das Trypsin durch Zugabe von DMEM-Medium mit 10% FBS neutralisiert (ca. 6,5 ml pro 6 cm Schale; dieses Medium entspricht dem oben beschriebenen Medium, enthält aber nur 10% FBS und kein LIF). Diese Suspension wurde dann auf 8 unbeschichtete Bakterienkultur-Petrischalen (Nunc Nr. 240045) verteilt und in einem Gesamtvolumen von ca. 4 ml DMEM/10% FBS pro 6 cm Schale weiter kultiviert.

1.4. Platieren der Embryoid Bodies

Nach 3-4 Tagen wurden die sich gebildeten Embryoid Bodies durch einfache (1 x g) Sedimentation für 5 min in einem 50 ml Zellkulturröhrchen gesammelt. Der Überstand wurde verworfen und die Embryoid Bodies derart auf 10 cm Zellkulturschalen verteilt, daß nach Absenken der Embryoid Bodies etwa 50% der Fläche der Zellkulturschale bedeckt waren (Embryoid Bodies aus ca. 4-6 Bakterienkulturschalen reichten in der Regel für die Beschickung einer 10 cm Zellkulturschale aus). Die Embryoid Bodies wurden über Nacht in DMEM/10% FBS (10 ml pro 10 cm Schale) kultiviert.

1.5. Transfer in ITSFn Medium

Am folgenden Tag wurden die mittlerweile adhärenten Embryoid Bodies 3 x in DMEM/F12 (Dulbecco's Modified Eagle Medium / Nutritient Mix F12 (1:1; Life Technologies, Nr. 11320)

Medium gewaschen. Anschließend wurden 10 ml ITSFn Medium pro 10 cm Schale zugegeben. ITSFn Medium besteht aus DMEM/F12, dem 5 µg/ml Insulin (Intergen Nr. 540101), 50 µg/ml Apo-Transferrin (Intergen Nr. 445275), 30 nM Seleniumchlorid (Sigma Nr. S-5261), 5 µg/ml Fibronektin (Life Technologies Nr. 33010) und Penicillin/Streptomycin (100 IU/ml / 100 µg/ml; Life Technologies Nr. 15140) zugesetzt wurden. Die Zellen wurden 4 - 7 Tage in diesem Medium kultiviert, wobei das Medium jeden zweiten Tag ersetzt wurde. Während der Kultivierung in ITSFn Medium kommt es zu massivem Zelltod unter den nicht-neuralen Zellen und zur Differenzierung in kleinen Grüppchen liegender neuraler Vorläuferzellen. Die hier beschriebene Etablierung von ITSFn Kulturen ist Stand der Technik (Okabe et al., Mech. Dev. 59:89-102, 1996).

1.6. Transfer in N3FL Medium

Die in ITSFn kultivierten Zellen wurden mittels einer Lösung von 0,05% Trypsin und 0,04% EDTA in PBS von der Kulturschale abgelöst und das Trypsin durch Zugabe einer äquivalenten Menge serumhaltigen Mediums (DMEM/10% FBS) neutralisiert. Nach Sedimentation (5 min, 300 x g, RT) wurden die Zellen in Calcium- und Magnesium-freier Hanks Buffered Salt Solution (CMF-HBSS, Life Technologies Nr. 14180) mit 0,1% DNase (Worthington Nr. 2139) resuspendiert (ca. 3 ml auf ein aus einer 10 cm Schale gewonnenes Pellet) und mit Hilfe von Pasteurpipetten zu einer Einzelzellsuspension trituriert. Zu diesem Zweck wurden mehrere Pasteurpipetten verwendet, deren Mündungen mit Hilfe einer Flamme abgerundet und verkleinert worden waren (auf 0,8 mm, 0,5 mm und 0,2 mm). Dabei wurde die Zellsuspension mehrere Male durch Pasteurpipetten mit abnehmender Mündungsgröße zu einer homogen-milchigen Suspension trituriert. Etwaige in der Suspension verbliebene Zellaggregate wurden vor Weiterbehandlung der Suspension durch Sedimentation (1 x g, 5 min) am Boden des Gefäßes gesammelt und verworfen. Die Zellen wurden dann zentrifugiert (300 x g, 5 min, RT) und in einer Dichte von ca. 30.000 Zellen/cm^2 auf zuvor mit Polyornithin beschichtete Zellkulturschalen in folgendem Medium ausgesät: DMEM/F12 (1:1; Life Technologies Nr. 11320), 25 µg/ml Insulin, 50 µg/ml humanes Apo-Transferrin, 20 nM Progesteron (Sigma Nr. P-8783), 100 µM Putrescin (Sigma Nr. P-5780), 30 nM Seleniumchlorid, 1 µg/ml Laminin (Life Technologies Nr. 23017), Penicillin/Streptomycin (100 IU/ml / 100 µg/ml), 10 ng/ml humanes rekombinantes bFGF (R&D Systems Nr. 233-FB). Dieses Medium entspricht dem von Okabe et al. geschilderten N3FL Medium (Okabe et al., Mech. Dev. 59:89-102, 1996). Für eine Polyornithin-Beschichtung wurden Zellkulturschalen mit einer Lösung von 15 µg/ml

Polyornithin (Sigma Nr. P-3655) in H_2O für mindestens 2 Stunden gefüllt. Anschließend wurde die Polyornithin-Lösung abgesaugt und die Kulturschale dreimal mit PBS gespült. bFGF wurde täglich zu einer Endkonzentration von 10 ng/ml zugesetzt und das Medium jeden zweiten Tag erneuert.

Beispiel 2: Gewinnung neuraler Vorläuferzellen

2.1. Transfer in N3EFL Medium

Nach 4-5 Tagen in diesem Medium wurden die Zellen unter Trypsin-freien Bedingungen mechanisch von der Kulturschale gelöst. Hierzu wurde die Kultur dreimal mit CMF-HBSS gespült, wobei der letzten Spülung 0,1% DNase zugesetzt wurden. Die Zellen wurden dann mit einem Zell-Lifter (Costar Nr. 3008) von der Platte abgeschabt und nach dem oben beschriebenen Modus mit Hilfe von Flammen-polierten Pasteur Pipetten zu einer Einzelzellsuspension trituriert. Nach Zentrifugation (300 x g, 5 min, RT) wurde der Ertrag einer 10 cm Zellkulturschale in ca. 5 zuvor mit Polyornithin beschichtete Zellkulturschalen in N3EFL Medium ausplatiert. Dieses setzte sich wie folgt zusammen: DMEM/F12 (1:1), 25 µg/ml Insulin, 100 µg/ml Transferrin, 20 nM Progesteron, 100 µM Putrescin, 30 nM Seleniumchlorid, 1 µg/ml Laminin, Penicillin/Streptomycin (100 IU/ml / 100 µg/ml), 10 ng/ml humanes rekombinantes bFGF und 20 ng/ml humanes rekombinantes EGF (R&D Systems Nr. 236-EG). bFGF und EGF wurden täglich zu einer Endkonzentration von 10 ng/ml für bFGF und 20 ng/ml für EGF zugesetzt. Das Medium wurde jeden zweiten Tag ersetzt, wobei Laminin nach dem zweiten Mediumwechsel nicht mehr zugesetzt wurde.

2.2. Transfer in N2FP Medium

Sobald die Zellen zu 90% konfluent geworden waren (nach 1-2 Wochen), wurden sie erneut unter Trypsin-freien Bedingungen mit einem Zell-Lifter mechanisch von der Kulturschale gelöst und mit Hilfe Flammen-polierter Pasteur Pipetten zu einer Einzelzellsuspension trituriert. Nach Zentrifugation (300 x g, 5 min, RT) wurde der Ertrag einer 10 cm Zellkulturschale in ca. 5 zuvor mit Polyornithin beschichtete Zellkulturschalen in N2FP Medium ausplatiert. Dieses setzte sich wie folgt zusammen: DMEM/F12 (1:1), 25 µg/ml Insulin, 100 µg/ml Transferrin, 20 nM Progesteron, 100 µM Putrescin, 30 nM Seleniumchlorid, Penicillin/Streptomycin (100 IU/ml / 100 µg/ml), 10 ng/ml humanes rekombinantes bFGF und 10 ng/ml rekombinantes humanes PDGF-AA (R&D Systems Nr.

221-AA). bFGF und PDGF-AA wurden täglich zugesetzt und das Medium jeden zweiten Tag erneuert.

2.3. Passagieren in N2FP Medium

Sobald die Zellen subkonfluent geworden waren, wurden sie trypsinfrei nach dem oben beschriebenen Modus 1:5 passagiert. Eine Triturierung der Zellen in DNase-haltiger Pufferlösung war hier meist entbehrlich. Nach mindestens zwei Passagen in diesem bFGF und PDGF-haltigen Medium bestand die Population aus neuralen Vorläuferzellen wie sie für remyelinisierende Transplantate verwendet werden konnten. Alternativ konnten die Zellen in diesem Stadium trypsinfrei von der Kulturschale gelöst und in serumfreiem Gefriermedium (Sigma Nr. C-6295) für eine spätere Verwendung tiefgefroren werden. Die gereinigten neuralen Vorläuferzellen in dem Medium können isoliert und in ein Injektionsmedium z.B. in Calcium- und Magnesium-freier Hanks Buffered Salt Solution (CMF-HBSS, Life Technologies Nr. 14180) aufgenommen werden.

2.4. *In vitro* Differenzierung der neuralen Vorläuferzellen

Für die *in vitro* Differenzierung der neuralen Vorläuferzellen wurden diese nach trypsinfreiem Passagieren in mit Polyornithin beschichtete Zellkulturschalen zu 50%iger Konfluenz ausplattiert und in dem unter Beispiel 2.2 genannten Medium, jedoch ohne bFGF und PDGF, für weitere 4-7 Tage kultiviert, wobei das Medium jeden zweiten Tag erneuert wurde. Die Kulturen wurden dann in 4% Paraformaldehyd-Lösung (Sigma Nr. P-6148) fixiert und einer Immunfluoreszenz-Untersuchung mit Antikörpern gegen das oligodendrogliale Antigen O4 (Boehringer Nr. 1518925, Verdünnung 1:10) und das astrozytäre Antigen GFAP (Chemicon, Nr. AB1980, Verdünnung 1:100) unterzogen. Hierbei zeigten bereits 4 Tage nach Wachstumsfaktorentzug bis zu 32% der Zellen eine typisch oligodendrogliale Morphologie mit Expression von O4; bis zu 49% der Zellen exprimierten zu diesem Zeitpunkt GFAP. Darüber hinaus fanden sich Zellen, welche das neuronale Antigen β-III-Tubulin exprimierten (Antikörper von BAbCO, Nr. MMS-435P-250, Verdünnung 1:500).

Beispiel 3: Gewinnung neuraler Sphäroide und daraus etablierter neuraler Vorläuferzellen

3.1. Etablierung neuraler Sphäroide in N2EF Medium

Nach 4-5 Tagen in dem in Beispiel 1.6 genannten N3Fl Medium wurden die Zellen unter Trypsin-freien Bedingungen mechanisch von der Kulturschale gelöst. Hierzu wurde die Kultur

dreimal mit CMF-HBSS gespült, wobei der letzten Spülung 0,1% DNase zugesetzt wurden. Die Zellen wurden dann mit einem Zell-Lifter (Costar Nr. 3008) von der Platte abgeschabt und nach dem oben beschriebenen Modus mit Hilfe von Flammen-polierten Pasteur Pipetten zu einer Einzelzellsuspension trituriert. Nach Zentrifugation (300 x g, 5 min, RT) wurden die Zellen in einer Dichte von 1.200 - 12.000 Zellen/cm^2 in unbeschichteten Zellkulturschalen in folgendem Medium ausgesät: DMEM/F12 (1:1), 25 µg/ml Insulin, 100 µg/ml Transferrin, 20 nM Progesteron, 100 µM Putrescin, 30 nM Seleniumchlorid, Penicillin/Streptomycin (100 IU/ml/100 µg/ml), 20 ng/ml humanes rekombinantes bFGF und 20 ng/ml humanes rekombinantes EGF (R&D Systems Nr. 236-EG). bFGF und EGF wurden täglich zu einer Endkonzentration von 20 ng/ml zugesetzt und das Medium (sog. N2EF Medium) alle zwei Tage erneuert. Hierzu wurde das verbrauchte Medium abgezogen und bei 150 x g für 3 min bei RT zentrifugiert, um etwaige im Medium schwimmende Sphäroide zu sedimentieren. Das Pellet wurde in frischem Medium resuspendiert und wieder ausgesät. Nach dem zweiten Mediumwechsel wurden die Zellen in frischen unbeschichteten Zellkulturschalen ausgesät.

3.2. *In vitro* Differenzierung der gewonnenen neuralen Sphäroide

Fünf Tage alte, gemäß Beispiel 3.1 erhaltene neurale Sphäroide wurden bei 150 x g für 3 min bei RT zentrifugiert und in dem in Beispiel 3.1 genannten Medium, jedoch ohne bFGF und EGF, in mit Polyornithin und Fibronektin beschichteten Zellkulturschalen ausgesät. Für eine Doppelbeschichtung von Zellkulturschalen mit Polyornithin und Fibronektin wurden die Platten zunächst in der unter Beispiel 1.6 beschriebenen Weise mit Polyornithin beschichtet und dreimal mit PBS gespült. Dann wurden die Schalen mit einer Lösung von 1 µg Fibronektin / ml PBS beschickt und für 2-12 Stunden bei RT inkubiert. Die Fibronektin-Lösung wurde abgezogen und die Zellsuspension in die derartig beschichtete Schale ausgesät. Einen Tag nach Aussaat hatten sich die neuralen Sphäroide an die Oberfläche der Zellkulturschale angeheftet. Zu diesem Zeitpunkt wurde das Medium abgezogen, die Schale dreimal mit frischem Medium oder CMF-HBSS gewaschen, und neues Medium eingefüllt, welches im weiteren alle zwei Tage ersetzt wurde. Derartig differenzierte neurale Sphäroid-Kulturen wurden 5 Tage nach Plattieren fixiert und einer Immunfluoreszenzanalyse unterzogen. Hierbei verteilte sich die Expression neuraler Antigene wie folgt (Mittelwert der Zahl gefärbter Zellen ± SEM): Nestin-positive neurale Vorläuferzellen 66±3% (Antikörper von M. Marvin und R.D.G. McKay, NIH, Behtesda, USA, Verdünnung 1:1.000), β-III-Tubulin-positive Neurone 34±3% (Antikörper von BAbCO, Nr. MMS-435P-250, Verdünnung 1:500), GFAP-positive

Astrozyten 30±2% (Antikörper von Chemicon, Nr. AB1980, Verdünnung 1:100), O4-positive Zellen mit oligodendroglialer Morphologie 6.2±1.7% (Antikörper von Boehringer, Nr. 1518925, Verdünnung 1:10), keines dieser Antigene aufweisende Zellen 2±0.7%. Weiter ließen sich in diesen für 5 Tage differenzierten Sphäroid-Kulturen in den entstandenen Nervenzellen Microtubule-Associated Protein 2 (MAP-2, Antikörper von Sigma, Nrs. M 4403 und M 1406, Verdünnung 1:200) und die Neurotransmitter GABA (Antikörper von Sigma, Nr. A-2052, Verdünnung 1:700) und Glutamat (Antikörper von Sigma, Nr. G-6642; Verdünnung 1:700) nachweisen. Der Anteil der GABA-positiven Neurone lag in manchen Präparationen bei bis zu 60%.

Wurden die Sphäroid-Kulturen über einen Zeitraum von 10 Tagen und mehr in Abwesenheit von Wachstumsfaktoren differenziert, liessen sich in den aus den Sphäroiden entstandenen Neuronen in Immunfluoreszenz-Untersuchungen zusätzlich Tyrosinhydroxylase (Antikörper von Eugene, Nr. TE101, Verdünnung 1:200), Cholinacetyltransferase (Antikörper von Chemicon, Nr. MAB305, Verdünnung 1:250), Serotonin (Antikörper von Eugene, Nr. NT102, Verdünnung 1:200), Synapsin (Antikörper von Dr. M. B. Kennedy, Pasadena, CA, USA, Verdünnung 1:1.000), Peripherin (Antikörper von Chemicon, Nr. AB1530, Verdünnung 1:1.000) und Calbindin (Antikörper von Sigma, Nr. C-8666, Verdünnung 1:100) nachweisen. Hierbei liessen sich Differenzierung und Überleben der Nervenzellen durch Zugabe der Neurotrophine Brain-Derived Neurotrophic Factor (BDNF; 20 ng/ml; Pepro Tech Inc. Nr. 450-02) und/oder Neurotrophin 3 (NT-3; Pepro Tech Inc. Nr. 450-03) steigern. In solchen über einen Zeitraum von 10 Tagen und mehr differenzierten Sphäroid-Kulturen kam es auch zu einer weiteren Ausreifung der Oligodendrozyten, in welchen sich dann in Immunfluoreszenz-Untersuchungen *cyclic nucleotide 3'-phosphodiesterase* (CNPase; Antikörper von Sigma, Nr. C-5922, Verdünnung 1:200) nachweisen liess.

3.3. Gewinnung glialer Vorläuferzellen aus neuralen Sphäroiden (sog. Touch-Down Kulturen)

Zur Gewinnung glialer Vorläuferzellen wurden die gemäß Beispiel 3.1 gewonnenen Sphäroide solange in dem unter Beispiel 3.1 genannten Wachstumfaktor-haltigen Medium kultiviert, bis sie begannen, spontan an die unbeschichtete Oberfläche der Zellkulturschalen zu adhärieren und Zellen aus den Sphäroiden auf die Oberfläche der Zellkulturschale ausgewandert waren. Dies war in der Regel nach einem Zeitraum von 10-14 Tagen der Fall. Sobald ein „Ring" ausgewanderter Zellen sichtbar wurde, wurden die der Zellkulturschale nur sehr locker

anhaftenden Sphäroide durch Schütteln der Schale losgelöst und mittels einer Pipette aus der Kultur entfernt. Die so entfernten Sphäroide konnten in weitere unbeschichtete Zellkulturschalen transferiert und im selben Medium weiter gezüchtet werden, wobei sie erneut „Ringe" auswachsender glialer Vorläuferzellen erzeugten. Die in den unbeschichteten Zellkulturschalen zurückbleibenden glialen Vorläuferzellen wurden in dem Wachstumsfaktor-haltigen Medium weiter proliferiert und nach Erreichen einer etwa 80%igen Konfluenz trypsinfrei nach dem oben beschriebenen Modus 1:5 passagiert. So gewonnene gliale Vorläuferzellen zeigten in Immunfluoreszenz-Untersuchungen eine deutliche Expression des neuralen Antigens A2B5 (Antikörper von Boehringer, Nr. 1300 016, Verdünnung 1:200). Sie konnten in serumfreiem Gefriermedium (Sigma Nr. C-6295) in flüssigem Stickstoff kryokonserviert und zu einem späteren Zeitpunkt weiter proliferiert, transplantiert oder differenziert werden.

3.4. Steuerung der glialen Differenzierung durch Zugabe von Faktoren

Die Steuerung der glialen Differenzierung durch Zugabe einzelner Faktoren wurde wie folgt überprüft. Gemäß Beispiel 3.3 erhaltene, in der Anwesenheit von bFGF (20 ng/ml) und EGF (20 ng/ml) kultivierte gliale Vorläuferzellen wurden trypsinfrei passagiert und in mit Polyornithin beschichtete Zellkulturschalen ausgesät. Nach Erreichen einer etwa 50%igen Konfluenz wurden die Zellen für zwei Tage wie folgt behandelt.

1. Weiterkultivieren in EGF und bFGF-haltigem Medium
2. Weiterkultivieren in EGF und bFGF-haltigem Medium
3. Weiterkultivieren in EGF und bFGF-haltigem Medium unter zusätzlicher täglicher Zugabe von CNTF (Regeneron, Inc.; verwendete Endkonzentration 10 ng/ml)
4. Weiterkultivieren in EGF und bFGF-haltigem Medium unter zusätzlicher täglicher Zugabe von T3 (Sigma Nr. T 6397; verwendete Endkonzentration 3 ng/ml)

Nach 2 Tagen wurden in den Gruppen 2, 3 und 4 bFGF und EGF entzogen und die Zellen für weitere 5-7 Tage kultiviert, wobei das Medium etwa alle 2 Tage ersetzt wurde. Dann wurden alle Kulturen in 4% Paraformaldehyd-Lösung (Sigma Nr. P-6148) fixiert und einer Immunfluoreszenz-Untersuchung mit Antikörpern gegen das oligodendrogliale Antigen O4 und das astrozytäre Antigen GFAP unterzogen. Dabei ergaben sich folgende Mittelwerte (± SEM):

1.	O4-positive Zellen mit oligodendroglialer Morphologie:	< 1%
	GFAP-positive Zellen	< 1%
2.	O4-positive Zellen mit oligodendroglialer Morphologie:	8.2 ± 2.6%
	GFAP-positive Zellen	28 ± 6%
3.	O4-positive Zellen mit oligodendroglialer Morphologie:	2.6 ± 1.8%
	GFAP-positive Zellen	78 ± 2.5%
4.	O4-positive Zellen mit oligodendroglialer Morphologie:	17 ± 3.6%
	GFAP-positive Zellen	39 ± 5.7%

Diese Daten zeigen, daß die Differenzierung der aus ES Zellen erhaltenen glialen Vorläuferzellen mit CNTF in eine astrozytäre und mit T3 in eine oligodendrogliale Richtung gelenkt werden kann.

Beispiel 4: Transplantation oligodendroglialer/astrozytärer Vorläuferzellen

4.1. Herstellung einer Zellsuspension für die Transplantation

Die remyelinisierende Potenz dieser Zellen wurde durch folgendes Transplantationsexperiment überprüft: Subkonfluente Kulturen aus Beispiel 2.3, welche noch bis zum Vortag der Transplantation bFGF und PDGF erhalten hatten, wurden trypsinfrei (mit einem Zell-Lifter) von der Kulturschale gelöst, bei 300 x g über 5 min sedimentiert und in CMF-HBSS mit 0,1% DNase resuspendiert. Die Zellen wurden dann mit Flammen-polierten Pasteurpipetten zu einer Einzelzellsuspension trituriert, in einer Zählkammer gezählt und erneut sedimentiert. Sie wurden in einer Konzentration von ca. 50.000-100.000 Zellen/µl in CMF-HBSS, welche 2 g/L Glucose (Sigma Nr. G-7021) enthielt, resuspendiert und bis zur Transplantation (bis zu 4-6 Stunden) auf Eis gelagert.

4.2. Intraventrikuläre Transplantation ins embryonale Rattengehirn

Als Transplantatempfänger wurden embryonale myelindefiziente Sprague-Dawley Ratten (Ian Duncan, Department of Medical Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Drive West, Madison, Wisconsin 53706, USA) verwendet.

Die frühe Transplantation während der Embryonalzeit hat den Vorteil, daß es trotz des Xenotransplantats nicht zu einer Abstoßungsreaktion kommt. Die myelindefizienten Empfängertiere haben den Vorteil, daß von den Donorzellen gebildetes Myelin einfach nachweisbar ist. Die Xenotransplantation selbst hat den Vorteil, daß die transplantierten Zellen einfach und mit hoher Sicherheit mit Hilfe speziesspezifischer DNA Proben nachgewiesen werden können (Brüstle et al., Neuron 15:1275-1285, 1995). Die Technik der intrauterinen Transplantation ins embryonale Gehirn ist Stand der Technik (Brüstle et al., Neuron 15:1275-1285, 1995; Brüstle et al., aus: Current Protocols in Neuroscience, John Wiley, New York, 1997). Zur Transplantation wurden schwangere Ratten am Embryonaltag 16 oder 17 mit einer intraperitonealen Injektion von Ketamin® (80 mg/kg) und Xylazin® (10 mg/kg) betäubt. Das Abdomen wurde eröffnet, und die einzelnen Embryonen unter Transillumination mit einer Faseroptik-Lichtquelle dargestellt. Wie beschrieben (vgl. Brüstle et al., Current Protocols in Neuroscience, John Wiley, New York, 1997) wurde die Zellsuspension in eine feine Glaskapillare mit einem Mündungsdurchmesser von 50-100 µm aufgezogen und die Glaskapillare durch den Uterus und die Schädeldecke in den Seitenventrikel des Embryos eingeführt. 2-9 µl der Zellsuspension (entsprechend 100.000-900.000 Zellen) wurden dann in das Ventrikelsystem injiziert. Die Glaskapillare wurde entfernt und die Bauchdecken nach Injektion mehrerer oder aller Embryonen wieder chirurgisch verschlossen. Derartig operierte Tiere werden spontan geboren. Da es sich um eine X-chromosomal rezessiv vererbte Erkrankung handelt, sind durchschnittlich 50% der männlichen Nachkommen myelindefizient. Diese Tiere beginnen in der dritten Lebenswoche stark zu zittern und sterben in der Regel in der vierten Lebenswoche.

4.3. Histologische Analyse der Transplantatempfänger

Zum Nachweis der Myelinisierung wurden die Tiere in üblicher Weise in der dritten bis vierten Lebenswoche betäubt und durch eine transkardiale Perfusion mit einer 4%igen Paraformaldehydlösung (Sigma Nr. P-6148) in PBS in üblicher Weise fixiert. Die Gehirne wurden in üblicher Weise präpariert und über Nacht in der Fixationslösung bei 4°C nachfixiert. Im Anschluß daran wurden auf einem Vibratom 50 µm dicke Schnitte angefertigt. Die Donorzellen wurden mittels DNA *in situ* Hybridisierung mit einem DNA-Sondenmolekül für Maus Satelliten DNA nachgewiesen (Brüstle et al., Neuron 15:1275-1285, 1995). Von den Donorzellen gebildetes Myelin wurde über eine immunhistochemische Detektion von Myelinproteinen in Fortsätzen hybridisierter Zellen nachgewiesen. Hierzu wurden Antikörper

gegen basisches Myelinprotein (Boehringer Nr. 1118099) oder Proteolipid Protein (Ian Griffiths, Department of Veterinary Clinical Studies, University of Glasgow, Bearsden, Scotland) verwendet. Die Schnitte wurden anschließend einer DNA Hybridisierung mit einem DNA-Sondenmolekül für Maus-Satelliten-DNA (Hörz & Altenburger, Nucl. Acids Res. 9:683-696, 1981) unterzogen (Brüstle et al., Neuron 15:1275-1285, 1995). Diese Doppelmarkierung gestattete es, gebildetes Myelin eindeutig den transplantierten Zellen zuzuordnen. In solchen Experimenten ließ sich eine Einwanderung der in den Ventrikel implantierten Zellen in eine Vielzahl tel-, di- und mesencephaler Hirnregionen nachweisen (14 untersuchte Tiere). Hybridisierte Zellen fanden sich unter anderem in Cortex, Hippocampus, Septum, Striatum, Bulbus olfactorius, Thalamus, Hypothalamus, Tectum, Cerebellum, Corpus callosum, in der vorderen Commissur sowie im Tractus opticus und innerhalb des Sehnerven. Nach Transplantation ließen sich im Ventrikelsystem der Empfängertiere keine raumfordernden Ansammlungen von Donorzellen nachweisen. Von 35 operierten Embryonen zeigten 11 männliche Nachkommen die Symptome einer Myelindefizienz. Von diesen waren 8 erfolgreich intraventrikulär transplantiert worden. In 6 dieser 8 Tiere konnte eine von den Donorzellen ausgehende Myelinisierung nachgewiesen werden. Hierzu wurde eine Doppelmarkierung hybridisierter Zellen mit dem PLP- oder MBP-Antikörper verwendet. In den verbleibenden 2 Fällen war die Zahl der inkorporierten Zellen für eine Doppelmarkierung zu niedrig; ein Screening mit dem Maus-spezifischen Antikörper M2 (Zhou et al., J. Comp. Neurol. 292:320-330, 1990) zeigte jedoch auch in diesen Tieren in das Wirtsgewebe inkorporierte Gliazellen. Die Donorzell-vermittelte Myelinisierung war am ausgeprägtesten in Faserbahnen wie z.B. dem Corpus callosum, der vorderen Commissur und commissuralen Fasern des Tectums. Darüber hinaus waren jedoch auch an zahlreichen Stellen innerhalb der grauen Substanz myelinisierende Donorzellen nachweisbar, so z.B. in Cortex, Septum, Thalamus, Hypothalamus und Tectum. In 7 der 8 erfolgreich transplantierten myelindefizienten Tiere liessen sich auch von den ES-Zellen abstammende inkorporierte Astrozyten nachweisen. Diese wurden immunhistochemisch mit Antikörpern gegen die Maus-spezifischen Antigene M2 (Zhou et al., J. Comp. Neurol. 292:320-330, 1990) und M6 (Lund et al., Neurosci. Lett. 61:221-226, 1985) oder durch eine immunhistochemische Doppelmarkierung von mit der Maus-spezifischen DNA Probe markierten Zellen mit einem Antikörper gegen saures Gliafaserprotein (GFAP; ICN Nr. 69-110) nachgewiesen.

Beispiel 5: Transplantation neuraler Sphäroide

5.1. Transplantation neuraler Sphäroide im Ibotensäure-Läsionsmodell der Ratte

Um eine anatomische und funktionelle Rekonstitution neuronaler Zellen durch die verfahrensgemäß hergestellten neuralen Vorläuferzellen zu überprüfen, wurden gemäß Beispiel 3.1 aus der ES Zellinie J1 gewonnene, 5 Tage alte neurale Sphäroide in das Striatum von erwachsenen Sprague-Dawley Ratten implantiert, in denen zuvor durch stereotaktische Injektion des Neurotoxins Ibotensäure ein einseitiger Neuronenuntergang im Striatum induziert worden war. Die striatale Ibotensäure-Läsion ist Stand der Technik und das verwendete Läsionsprotokoll veröffentlicht (Brüstle et al., aus: Current Protocols in Neuroscience, Unit 3.10, John Wiley, New York, 1997). Für die Transplantation wurden das Medium und die darin schwimmenden Sphäroide der Zellkulturschale entnommen und in ein 50 ml Plastikröhrchen überführt. Nach Zugabe von DNase zu einer Gesamtkonzentration von 0.1% wurden die Späroide bei 150 x g über 5 min bei RT sedimentiert und dann in CMF-HBSS, welche 2 g/L Glucose (Sigma Nr. G-7021) enthielt, in einem 0.5 ml Plastikreaktionsröhrchen resuspendiert. Die so resuspendierten Sphäroide wurden bis zur Transplantation (bis zu 4 Stunden) auf Eis gelagert. Die Empfängertiere wurden in üblicher Weise betäubt und die Sphäroide über eine stereotaktische Injektion in das Striatum eingeführt. Für die Injektion wurde eine Glaskapillare mit einer Öffnung von etwa 0.25 - 0.75 mm verwendet. Das verwendete Transplantationsprotokoll ist Stand der Technik und ausführlich publiziert (Brüstle et al., aus: Current Protocols in Neuroscience, Unit 3.10, John Wiley, New York, 1997). Es wurden insgesamt 6 Tiere transplantiert. Dabei wurden die sich am Boden des 0.5 ml Röhrchens angesammelten Sphäroide in die Glaskapillare aufgezogen und mit Hilfe eines stereotaktischen Rahmens (Stoelting Nr. 51600) in das Striatum injiziert. Die Sphäroide wurden dabei über 2 (n=3) oder 5 (n=3) Stellen innerhalb des geschädigten Striatums verteilt. Hierbei wurden folgende stereotaktischen Daten verwendet:

Für 2 Implantationsorte:

Ort 1:	Frontzahnhalter:	- 2.3 mm
	Anteroposteriore Ausrichtung:	+ 0.2 mm (relativ zum Bregma)
	Mediolaterale Ausrichtung:	3.0 mm (relativ zu Sagittalnaht)
	Injektionstiefe	5.5 mm (relativ zur Dura)

Ort 2:	Frontzahnhalter:	- 2.3 mm
	Anteroposteriore Ausrichtung:	+ 0.2 mm (relativ zum Bregma)
	Mediolaterale Ausrichtung:	3.0 mm (relativ zu Sagittalnaht)
	Injektionstiefe	4.0 mm (relativ zur Dura)

Für 5 Implantationsorte:

Ort 1:	Frontzahnhalter:	- 2.3 mm
	Anteroposteriore Ausrichtung:	+ 0.2 mm (relativ zum Bregma)
	Mediolaterale Ausrichtung:	3.0 mm (relativ zu Sagittalnaht)
	Injektionstiefe	6.5 mm (relativ zur Dura)
Ort 2:	Frontzahnhalter:	- 2.3 mm
	Anteroposteriore Ausrichtung:	+ 0.2 mm (relativ zum Bregma)
	Mediolaterale Ausrichtung:	3.0 mm (relativ zu Sagittalnaht)
	Injektionstiefe	5.3 mm (relativ zur Dura)
Ort 3:	Frontzahnhalter:	- 2.3 mm
	Anteroposteriore Ausrichtung:	+ 0.2 mm (relativ zum Bregma)
	Mediolaterale Ausrichtung:	3.0 mm (relativ zu Sagittalnaht)
	Injektionstiefe	4.0 mm (relativ zur Dura)
Ort 4:	Frontzahnhalter:	- 2.3 mm
	Anteroposteriore Ausrichtung:	+ 1.5 mm (relativ zum Bregma)
	Mediolaterale Ausrichtung:	2.5 mm (relativ zu Sagittalnaht)
	Injektionstiefe	6.0 mm (relativ zur Dura)
Ort 5:	Frontzahnhalter:	- 2.3 mm
	Anteroposteriore Ausrichtung:	+ 1.5 mm (relativ zum Bregma)
	Mediolaterale Ausrichtung:	2.5 mm (relativ zu Sagittalnaht)
	Injektionstiefe	4.5 mm (relativ zur Dura)

Pro Injektionsort wurden 10 µl der Sphäroid Suspension implantiert.

Da es sich um Xenotransplantate handelte, wurden die Tiere einer Immunsuppression unterzogen. Hierzu erhielten die Empfängertiere täglich, beginnend 1 Tag vor Transplantation, eine intraperitoneale Injektion von 20 mg Cyclosporin A pro kg Körpergewicht (Sandimmun, Fa. Sandoz). Um opportunistische Infektionen zu vermeiden, wurde dem Trinkwasser während der Immunsuppressionsbehandlung Tetracyclin zugesetzt (Achromycin, Fa. Lederle; Endkonzentration 100 mg/L).

5.2. Analyse der funktionellen Verbesserung der Transplantatempfänger

Von den 6 operierten Tieren überlebten 4 Tiere mehr als 4 Wochen nach Transplantation. Bei diesen Tieren ergab die Untersuchung des Amphetamin-induzierten Rotationsverhaltens vor und nach Transplantation folgende Werte (Mittelwert der Rotationen pro Minute):

Tier 200:	34 Tage vor Transplantation:		9.7
	37 Tage nach Transplantation:	13.9	
Tier 201:	24 Tage vor Transplantation:		13.5
	37 Tage nach Transplantation:	1.3	
Tier 204:	43 Tage vor Transplantation:		12.7
	50 Tage nach Transplantation:	0.5	
Tier 205:	86 Tage vor Transplantation:		9.0
	42 Tage nach Transplantation:	1.0	

Zur Messung des Rotationsverhaltens wurde den Tieren intraperitoneal eine Dosis von 5 mg D-Amphetamin Sulfat (Sigma Nr. A-3278) pro kg Körpergewicht injiziert. Nach 5-minütiger Einwirkungszeit wurde die Zahl der ausgelösten Rotationen pro Minute über einen Zeitraum von 90 Minuten gemessen und gemittelt. Die Messung des Amphetamin-induzierten Rotationsverhalten als Parameter für die Funktion striataler Transplantate ist Stand der Technik und publiziert (Brüstle et al., aus: Current Protocols in Neuroscience, Unit 3.10, John

Wiley, New York, 1997). Die Daten zeigen, daß 3 der 4 untersuchten Tiere eine deutliche Reduktion des Rotationsverhaltens aufwiesen.

5.3. Histologische Analyse der Transplantatempfänger

Für die histologische Untersuchung der Transplantate wurden die Tiere 5-9 Wochen nach Transplantation betäubt und durch eine transkardiale Perfusion mit einer 4%igen Paraformaldehydlösung in PBS in üblicher Weise fixiert. Die Gehirne wurden in üblicher Weise präpariert und über Nacht in der Fixationslösung bei 4°C nachfixiert. Im Anschluß daran wurden auf einem Vibratom 50 µm Schnitte angefertigt. Die Donorzellen wurden mittels DNA *in situ* Hybridisierung mit einer Probe für Maus Satelliten DNA nachgewiesen (Hörz & Altenburger, Nucl. Acids Res. 9:683-696, 1981; Brüstle et al., Neuron 15:1275-1285, 1995). Dabei fanden sich in allen 4 der in den Verhaltenstests (Beispiel 5.2) untersuchten Tiere hybridisierte Zellen im Implantationsgebiet. Ein Teil der hybridisierten Zellen war zudem aus dem Implantationsgebiet in benachbarte Hirnabschnitte, besonders in das Corpus Callosum, eingewandert. Das Tier 200 wies einen sehr weiten Seitenventrikel und ein intraventrikulär lokalisiertes Transplantat auf. Offenbar war durch die vorausgegangene Ibotensäureläsion eine starke Atrophie des Striatums mit konsekutiver Ventrikelerweiterung entstanden und die nachfolgend implantierten Zellen landeten anstatt im Striatum im Seitenventrikel. Die inkorrekte Lage im Seitenventrikel erklärt auch die ausbleibende funktionelle Verbesserung dieses Empfängertieres in den Rotationstests.

Neben positivem Hybridisierungssignal wiesen die transplantierten Zellen eine deutliche Expression des Maus-spezifischen neuralen Antigens M6 auf. Dieses Antigen wird besonders auf Axonen exprimiert (Lund et al., Neurosci. Lett. 61:221-226, 1985; Antikörper von C. Lagenaur, Department of Neurobiology, University of Pittsburgh School of Medicine, 818A Scaife Hall, Pittsburgh, Pennsylvania 15261, USA, Verdünnung 1:10). In den Empfängertieren ließen sich zahlreiche M6-positive Axone erkennen, welche vom Transplantat ausgingen und in das umgebende Hirngewebe eingesproßt waren. Dies weist darauf hin, daß die aus den transplantierten Sphäroiden hervorgegangenen Nervenzellen das Empfängergehirn innervieren.

Patentansprüche

1. Isolierte, nach Transplantation in das Nervensystem nicht-tumorigene gereinigte Vorläuferzellen aus embryonalen Stammzellen mit neuronalen oder glialen Eigenschaften.

2. Isolierte, gereinigte nicht-tumorigene Vorläuferzellen mit neuronalen oder glialen Eigenschaften aus embryonalen Stammzellen, enthaltend höchstens etwa 15% primitive embryonale und nicht-neurale Zellen, erhältlich durch folgende Schritte:

a) proliferieren von ES-Zellen,

b) kultivieren der ES-Zellen aus Schritt a) zu neuralen Vorläuferzellen,

c) proliferieren der neuralen Vorläuferzellen in einem Wachstumsfaktor-haltigen serumfreien Medium,

d) proliferieren der neuralen Vorläuferzellen aus Schritt c) in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium und isolieren der gereinigten neuralen Vorläuferzellen und

e) proliferieren der neuralen Vorläuferzellen aus Schritt d) in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium und isolieren der gereinigten Vorläuferzellen mit neuronalen oder glialen Eigenschaften.

3. Zellen nach Anspruch 2, wobei die ES-Zellen in Schritt a) zu Zellaggregaten, insbesondere Embryoid Bodies proliferiert werden.

4. Isolierte, gereinigte nicht-tumorigene Vorläuferzellen mit neuronalen oder glialen Eigenschaften aus embryonalen Stammzellen, enthaltend höchstens etwa 15% primitive embryonale und nicht-neurale Zellen, erhältlich durch folgende Schritte:

a') proliferieren von ES-Zellen,

b') kultivieren der ES-Zellen aus Schritt a') zu neuralen Vorläuferzellen,

c') proliferieren der neuralen Vorläuferzellen in einem Wachstumsfaktor-haltigen serumfreien Medium,

d') proliferieren der neuralen Vorläuferzellen aus Schritt c') in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium zu neuralen Sphäroiden und isolieren der neuralen Sphäroide und

e') proliferieren der neuralen Sphäroide aus Schritt d') in einem Wachstumsfaktor-haltigen serumfreien Medium bis zur Ausbildung eines aus glialen Vorläuferzellen bestehenden Zellrasens und isolieren der gereinigten Vorläuferzellen mit glialen Eigenschaften.

5. Zellen nach Anspruch 4, wobei die ES-Zellen in Schritt a') zu Zellaggregaten, insbesondere Embryoid Bodies proliferiert werden.

6. Zellen nach einem der Ansprüche 1 bis 5 in Form eines Zellrasens.

7. Zellen nach einem der Ansprüche 1 bis 5 in Form von Sphäroiden.

8. Zellen nach einem der Ansprüche 1 bis 5, umfassend Zellen mit neuronalen, astrozytären und/oder oligodendroglialen Eigenschaften.

9. Zellen nach einem der Ansprüche 1 bis 5, wobei die embryonalen Stammzellen aus Oocyten nach einer Kerntransplantation erhalten worden sind.

10. Zellen nach einem der Ansprüche 1 bis 5, wobei die embryonalen Stammzellen aus embryonalen Keimzellen erhalten worden sind.

11. Zellen nach einem der Ansprüche 1 bis 5, wobei die Zellen Säugerzellen sind.

12. Zellen nach Anspruch 11, wobei die Zellen aus der Gruppe umfassend Maus, Ratte, Hamster, Schaf, Schwein, Rind, Primaten oder Mensch isoliert worden sind.

13. Zellen nach einem der Ansprüche 1 bis 10, wobei die Zellen gentechnisch modifiziert worden sind.

14. Zellen nach einem der Ansprüche 1 bis 13 in tiefgefrorener Form.

15. Zellbibliothek, umfassend autologe und nicht autologe Zellen nach einem der Ansprüche 1 bis 14.

16. Sphäroide, umfassend differenzierte neurale Zellen der Vorläuferzellen nach einem der Ansprüche 1 bis 5.

17. Sphäroide nach Anspruch 16, dadurch erhalten, daß die Vorläuferzellen nach einem der Ansprüche 1 bis 5 *in vitro* zur Differenzierung induziert worden sind.

18. Verfahren zur Herstellung von gereinigten Vorläuferzellen mit neuronalen oder glialen Eigenschaften, umfassend die folgenden Schritte:
a) proliferieren von ES-Zellen,
b) kultivieren der ES-Zellen aus Schritt a) zu neuralen Vorläuferzellen,
c) proliferieren der neuralen Vorläuferzellen in einem Wachstumsfaktor-haltigen serumfreien Medium,
d) proliferieren der neuralen Vorläuferzellen aus Schritt c) in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium und isolieren der gereinigten neuralen Vorläuferzellen und
e) proliferieren der neuralen Vorläuferzellen aus Schritt d) in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium und isolieren der gereinigten Vorläuferzellen mit neuronalen oder glialen Eigenschaften.

19. Verfahren nach Anspruch 18, wobei die ES-Zellen in Schritt a) zu Zellaggregaten, insbesondere Embryoid Bodies proliferiert werden.

20. Verfahren nach Anspruch 18 oder 19, wobei das Wachstumsfaktor-haltige serumfreie Medium in Schritt c) bFGF umfaßt.

21. Verfahren nach einem der Ansprüche 18 bis 20, wobei das Wachstumsfaktor-haltige serumfreie Medium in Schritt d) bFGF und EGF umfaßt.

22. Verfahren nach einem der Ansprüche 18 bis 21, wobei das Wachstumsfaktor-haltige serumfreie Medium in Schritt e) bFGF und PDGF umfaßt.

23. Verfahren zur Herstellung von gereinigten Vorläuferzellen mit neuronalen oder glialen Eigenschaften, umfassend die folgenden Schritte:
a') proliferieren von ES-Zellen,
b') kultivieren der ES-Zellen aus Schritt a') zu neuralen Vorläuferzellen,
c') proliferieren der neuralen Vorläuferzellen in einem Wachstumsfaktor-haltigen serumfreien Medium,
d') proliferieren der neuralen Vorläuferzellen aus Schritt c') in einem weiteren Wachstumsfaktor-haltigen serumfreien Medium zu Sphäroiden mit neuronalen und glialen Differenzierungspotential und isolieren der Sphäroide und
e') proliferieren der neuralen Sphäroide aus Schritt d') in einem Wachstumsfaktor-haltigen serumfreien Medium bis zur Ausbildung eines aus glialen Vorläuferzellen bestehenden Zellrasens und isolieren der gereinigten Vorläuferzellen mit glialen Eigenschaften.

24. Verfahren nach Anspruch 23, wobei die ES-Zellen in Schritt a') zu Zellaggregaten, insbesondere Embryoid Bodies proliferiert werden.

25. Verfahren nach Anspruch 23 oder 24 ferner umfassend den Schritt:
f') Steuerung der Differenzierung der glialen Vorläuferzellen aus Schritt e') in eine astrozytäre oder in eine oligodendrogliale Richtung und isolieren der Vorläuferzellen mit astrozytären oder oligodendroglialen Eigenschaften.

26. Verfahren nach einem der Ansprüche 23 bis 25, wobei das Wachstumsfaktor-haltige serumfreie Medium in Schritt c') bFGF umfaßt.

27. Verfahren nach einem der Ansprüche 23 bis 26, wobei das Wachstumsfaktor-haltige serumfreie Medium in Schritt d'), e') und f') bFGF und/oder EGF umfaßt.

28. Verfahren nach einem der Ansprüche 25 bis 27, wobei das Medium in Schritt f') entweder CNTF oder T3 umfaßt.

29. Verfahren nach einem der Ansprüche 18 bis 28, wobei das Verfahren mit Zelltrennungs- und Sortierverfahren kombiniert wird.

30. Verfahren nach einem der Ansprüche 18 bis 29, wobei man die gereinigten Vorläuferzellen in einem Injektionsmedium aufnimmt.

31. Verwendung der Vorläuferzellen nach einem der Ansprüche 1 bis 15 zur Transplantation in das Nervensystem.

32. Verwendung der Sphäroide nach Anspruch 16 oder 17 zur Transplantation in das Nervensystem.

33. Verwendung der Vorläuferzellen nach einem der Ansprüche 1 bis 15 zur Therapie von neuralen Defekten.

34. Verwendung der Vorläuferzellen nach einem der Ansprüche 1 bis 15 zur Rekonstitution neuronaler Zellen.

35. Verwendung der Vorläuferzellen nach einem der Ansprüche 1 bis 15 zur Remyelinisierung demyelinisierter Bereiche des Nervensystems.

36. Verwendung der Vorläuferzellen nach einem der Ansprüche 1 bis 15 für zellvermittelten Gentransfer ins Nervensystem.

37. Verwendung der Vorläuferzellen nach einem der Ansprüche 1 bis 15 zur *in vitro* Gewinnung von Polypeptiden.

38. Arzneimittelzusammensetzung, enthaltend Vorläuferzellen nach einem der Ansprüche 1 bis 15 zur Therapie von neuralen Defekten.

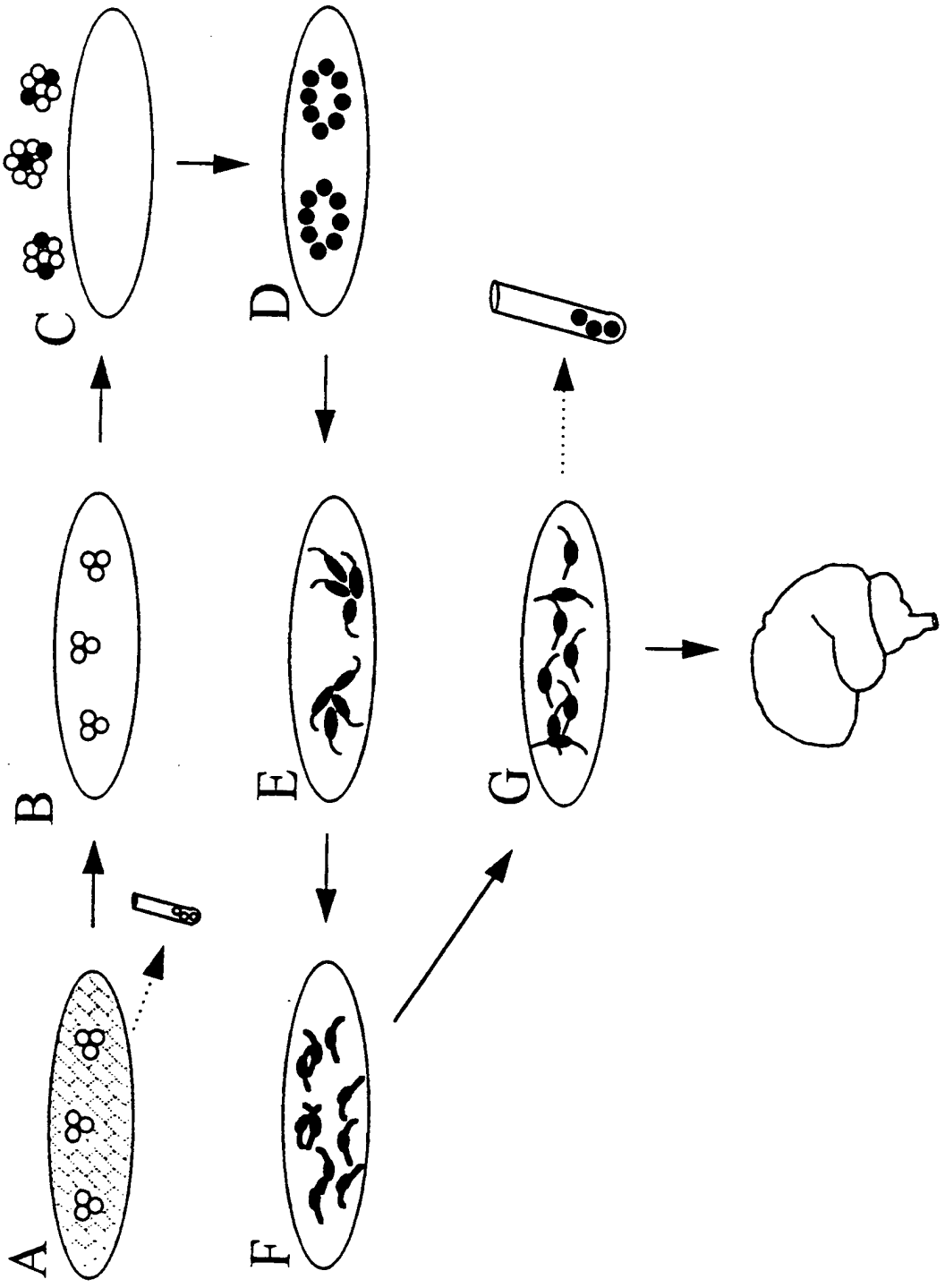


Fig. 1

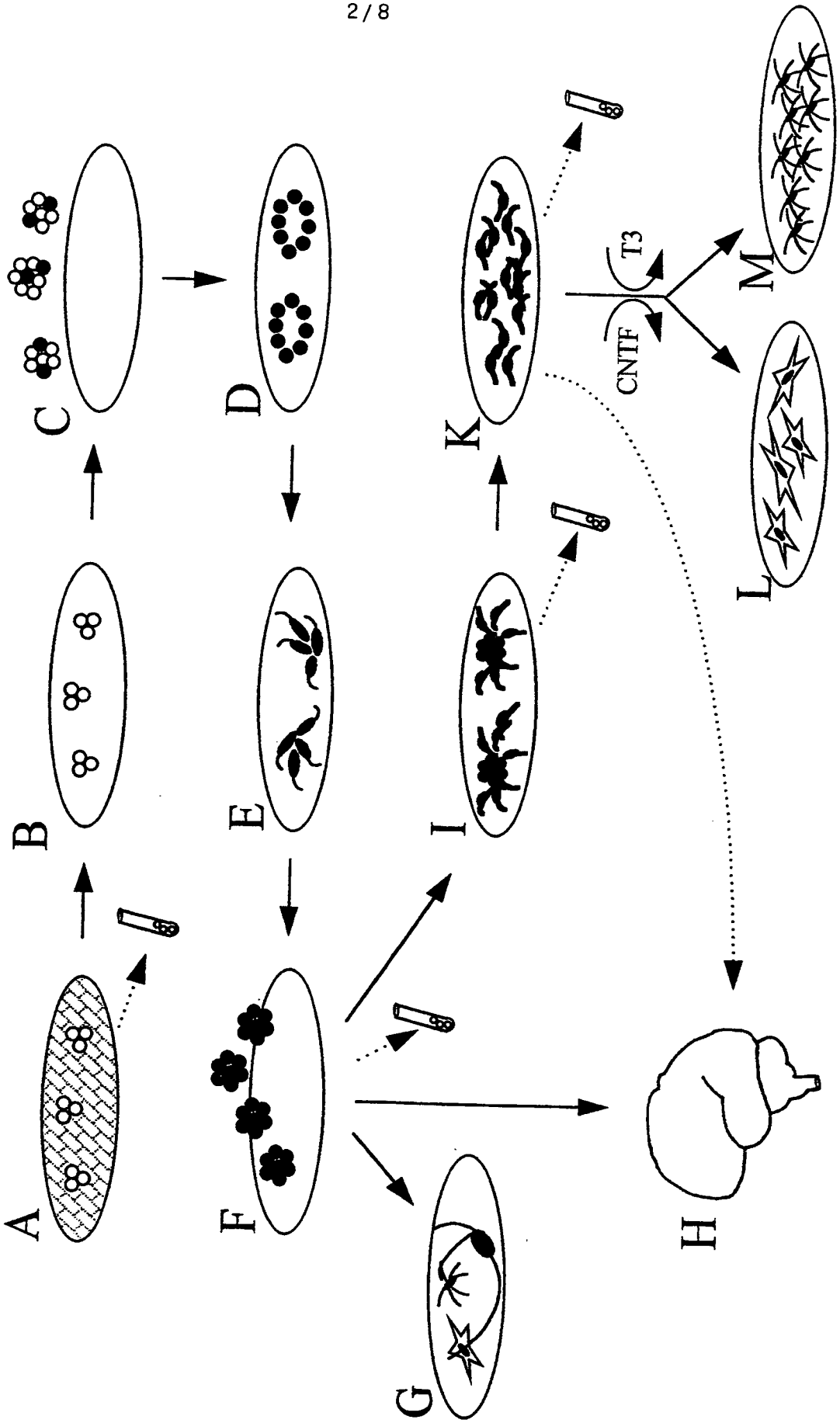


Fig. 2

Fig. 3

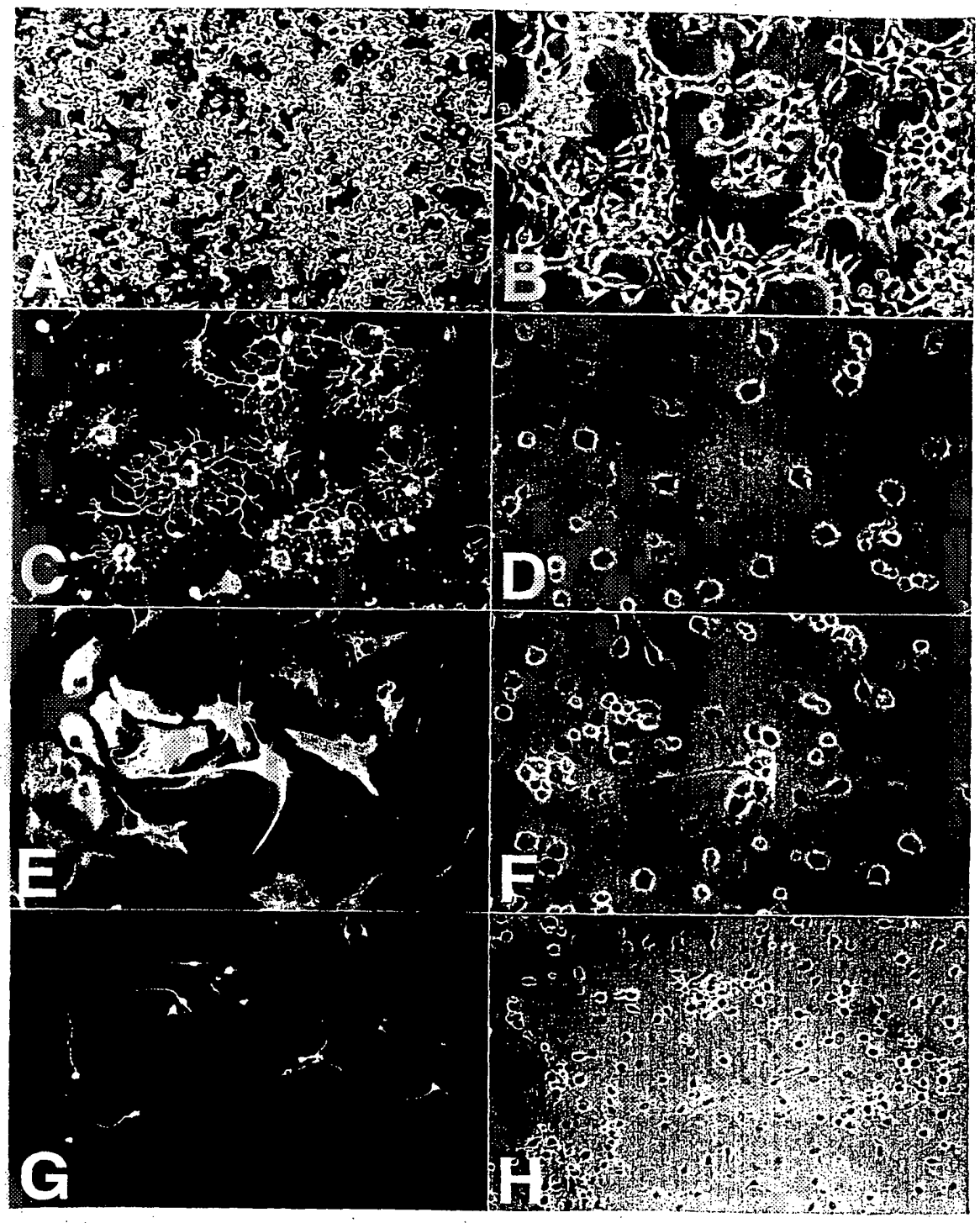


Fig. 4

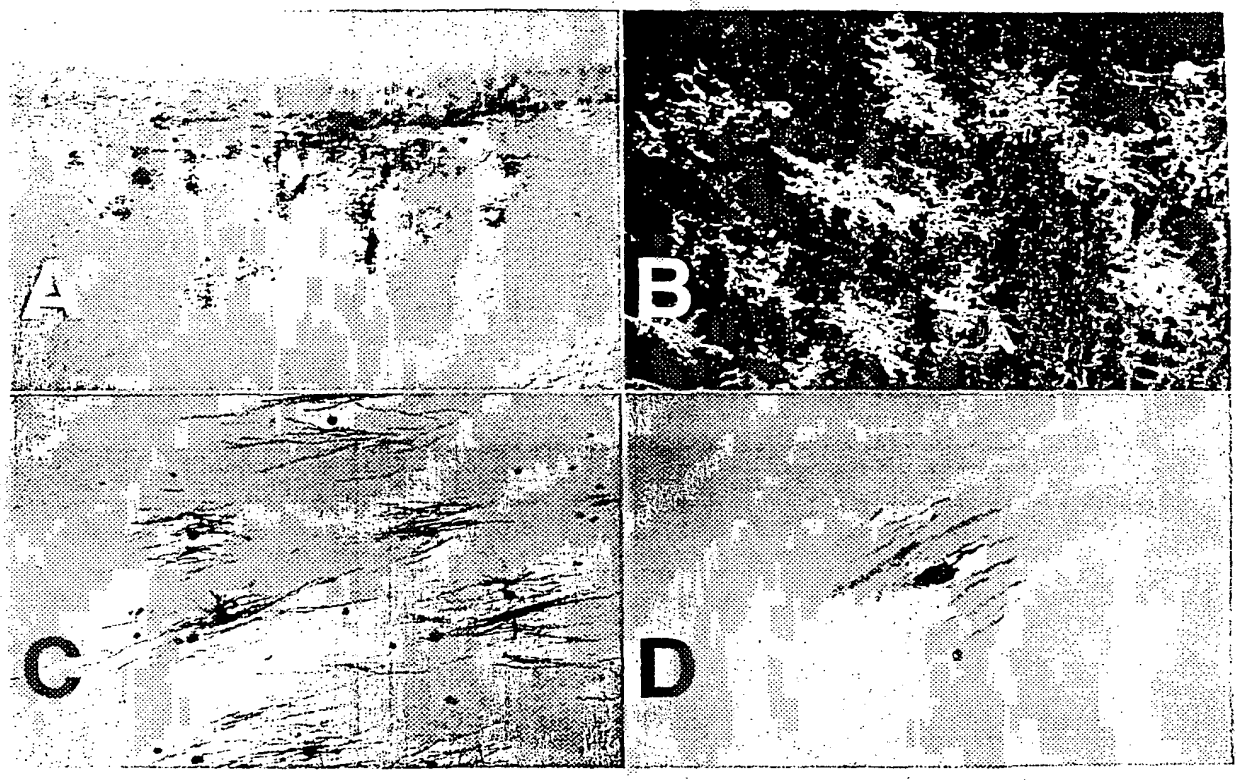


Fig. 5

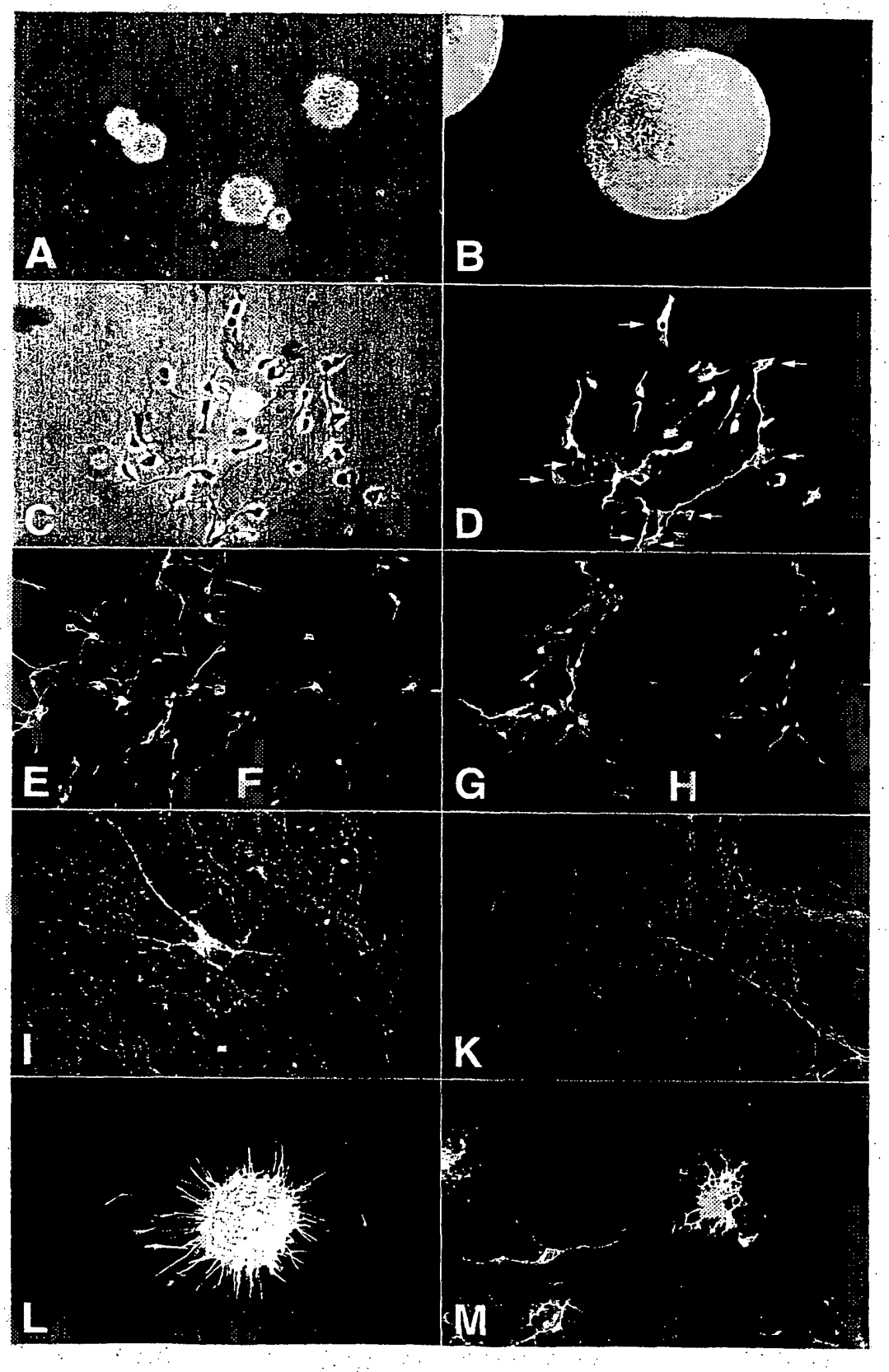


Fig. 6

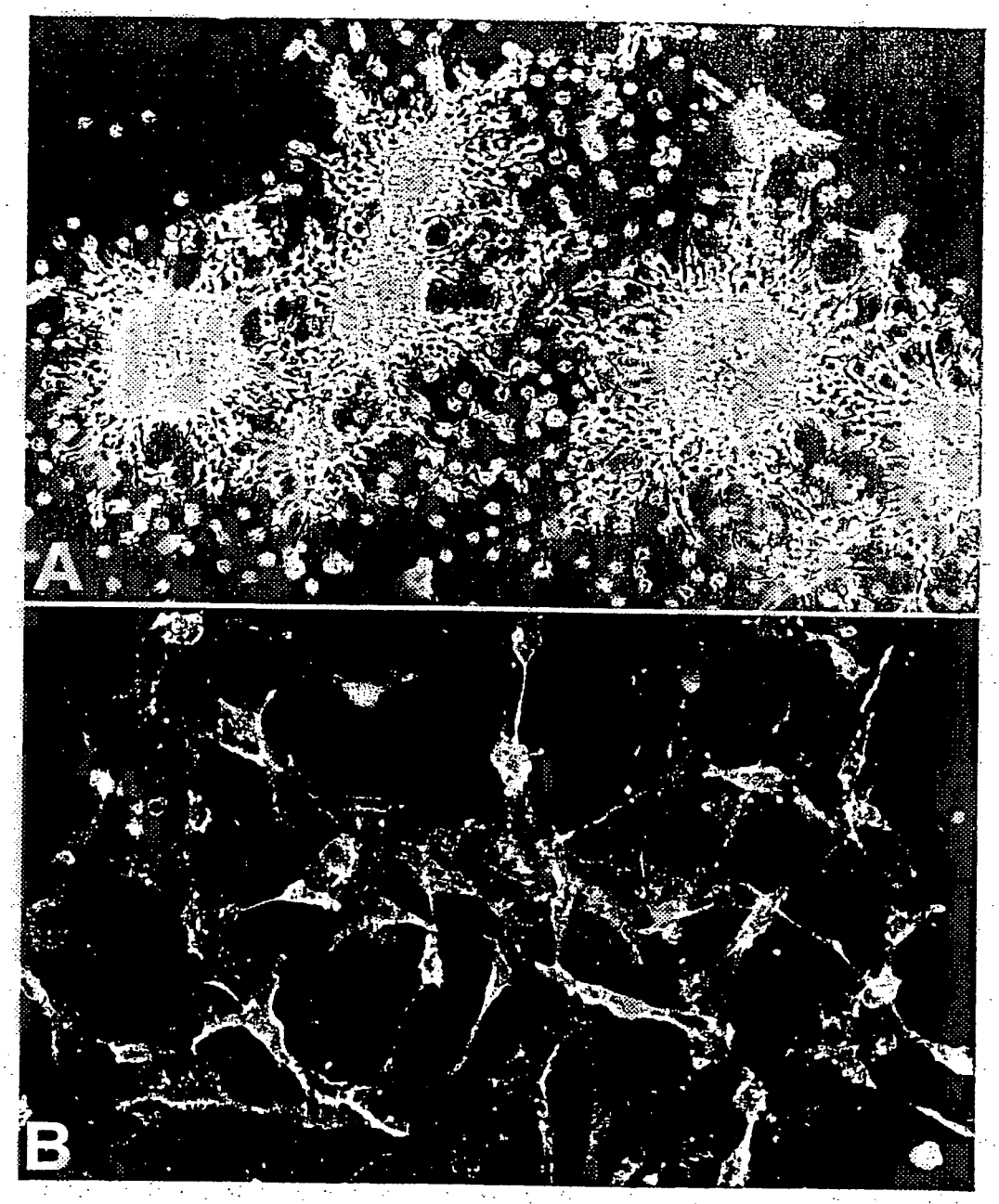


Fig. 7

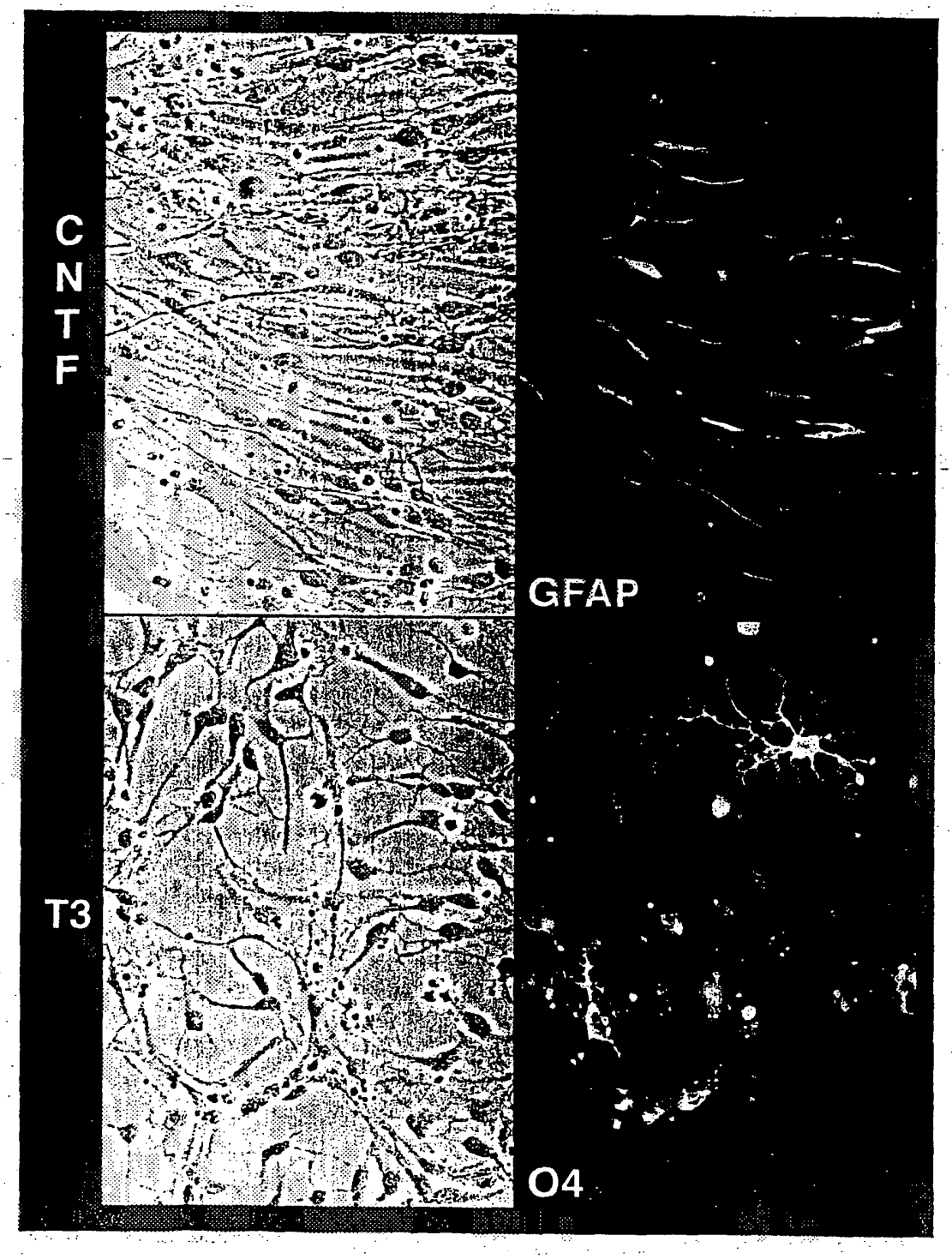
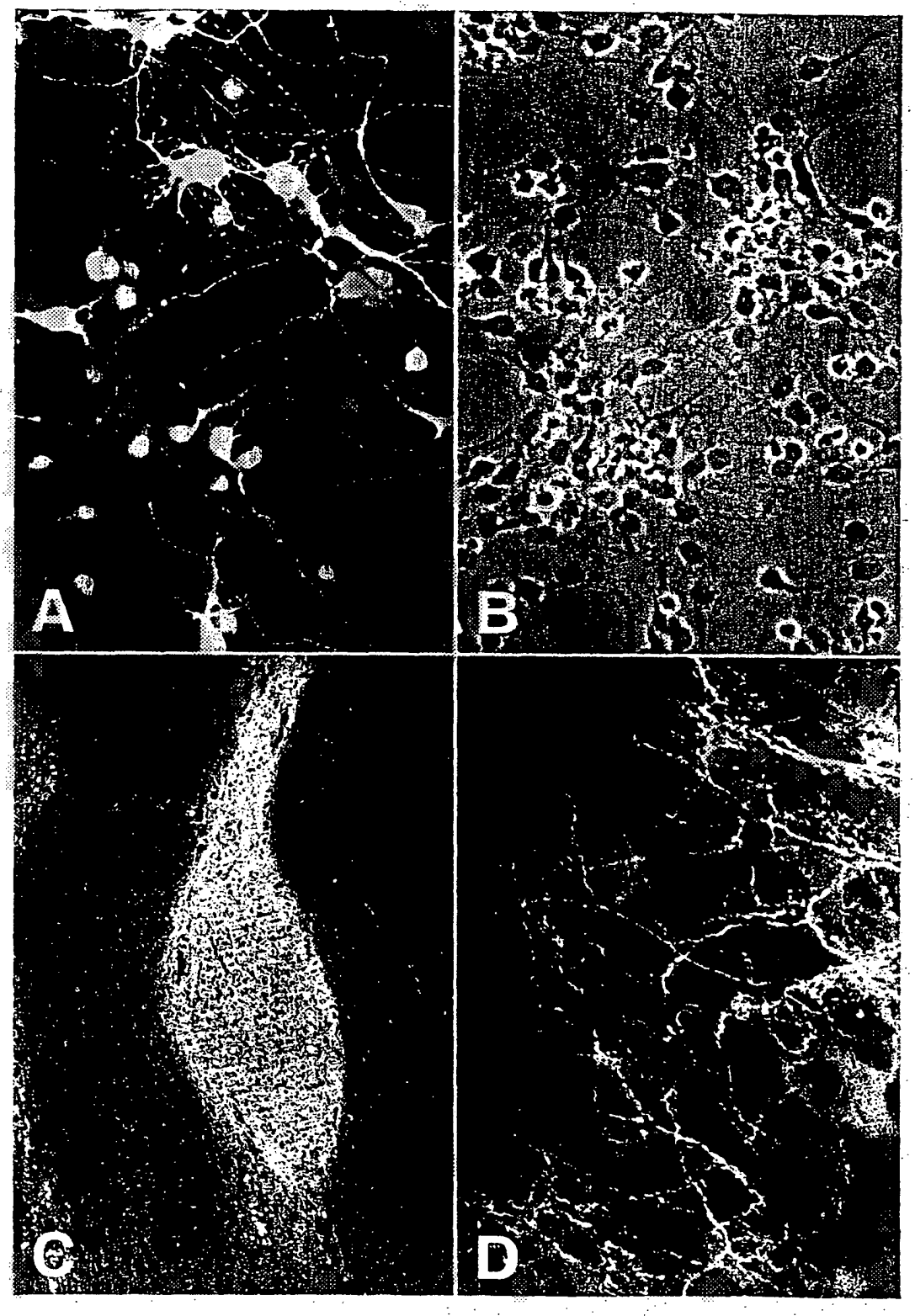


Fig. 8



PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/06, 5/08, A61K 35/30	A1	(11) International Publication Number: WO 97/16534
		(43) International Publication Date: 9 May 1997 (09.05.97)

(21) International Application Number: PCT/US96/16543

(22) International Filing Date: 15 October 1996 (15.10.96)

(30) Priority Data:
08/548,345 1 November 1995 (01.11.95) US

(71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

(72) Inventors: LI, Ronghao; 401 Richmond Drive #203, Millbrae, CA 94030 (US). MATHER, Jennie; 269 La Prenda Avenue, Millbrae, CA 94030 (US).

(74) Agents: KUBINEC, Jeffrey, S. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NORMAL NEURAL EPITHELIAL PRECURSOR CELLS

(57) Abstract

A method for isolating normal neural epithelial cells in culture is provided wherein embryonic neural tissue is cultured in the presence of embryonic Schwann cells or embryonic Schwann cell conditioned medium. A normal neural epithelial precursor cell line capable of long term culture is also provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

PNSDOCID: <WO_9716534A1>

NORMAL NEURAL EPITHELIAL PRECURSOR CELLS

Field of the Invention

The present invention relates to methods for isolating normal neural epithelial precursor cells that are capable of cell replication, expansion, and long term culture in vitro while maintaining their ability for differentiation in vitro and in vivo. The methods for isolating the normal neural epithelial precursor cell type of the present invention result in a substantially homogeneous population of non-transformed, non-tumorigenic neural epithelial precursor cells. Therefore, the invention also relates to normal neural epithelial precursor cell lines of mammalian origin. The isolated neural epithelial precursor cells of the invention can be maintained and expanded in culture employing the methods described herein.

Description of Related Disclosures

The mammalian central nervous system is developmentally derived from neuroepithelial cells in the neural tube (Murphy, M. et al., J. Neurosci. Res. (1990) 25:463-475; see generally,"Developmental Biology" Fourth Edition, Gilbert, S., (1994) Sinauer Associates, Inc.; "Post Implantation Mammalian Embryos: A Practical Approach", Copp, A.J., and Cockroft, D.L., eds. (1990) Oxford University Press, New York). In vivo, neuroepithelial cells grow and differentiate to different types of neuronal and glial cells in response to, as yet unidentified, environmental factors. The ability to isolate and maintain particular neural precursor cells in vitro is crucial to the identification of these factors. Additionally, the ability to isolate and expand non-tumorigenic neural cell types at early embryonic stages of development is critical to the success of cell based therapies including intracerebral grafting and gene transfer (Gage et al., (1991), TINS, 14:8328-333; Mucke and Rockenstein, (1993) Transgene, 1:3-9; Jiao et al., (1992) Brain Res. 575:143-147).

Broadly speaking, there are two types of cell culture for the maintenance and characterization of cells of the mammalian nervous system (Bottenstein, J.E., "Growth and Differentiation of Neural Cells in Defined Media." Cell Culture in the Neurosciences, Bottenstein and Sato, eds., pp 3-43 [1985]). Primary culture involves tissue explant and subsequent culture of either the dispersed cells or the explanted tissue for the finite lifetime of the dispersed cells or tissue. After several days in culture the cells generally spontaneously differentiate into a variety of neuronal cell types (Cattaneo and McKay (1990) Nature 347:762-765). The primary advantage of dissociated cell culture is that single cell types can be studied as representative of actual in vivo cell types ("Developmental Biology of Cultured Nerve, Muscle, and Glia," David Shubert, John Wiley & Sons, Inc. (1984)). Using primary culture heterogenous samples of cells can be isolated to achieve representative cell populations of uniform phenotype. Many populations of cells derived from explanted tissue have, heretofore, been limited in the number of times they can divide possibly owing to in vitro aging (Orgel, L.E. (1973) Nature, 243:441-445) or inappropriate culture conditions.

Continuous cell culture represents a second variety of in vitro cell culture for cells of the developing mammalian central nervous system (Bottenstein, J. (1985) supra). Continuous cell culture provides the advantage of long term culture and storage. Since the proliferation and differentiation of neuroepithelial cells is a programmed process in vivo and neuroepithelial cells cultured in vitro tend to differentiate spontaneously after limited divisions, long term culture of neuroepithelial cells has previously relied on viral or oncogene transformation.

Several groups have reportedly established multipotent neural cell lines through retroviral gene transfer (Snyder, et al., Cell, (1992) 68:33-51; Ryder et al., J. Neurobiol. (1990) 21:356-375; Geller and Dubois-Dalq, (1988) J. Cell Biol. 107:1977-1986; Bartlett, (1988) Proc. Natl. Aced. Sci. USA 85:3255-3259; Birren and Anderson (1990) Neuron 4:189-201; Evrad, et al., (1990) Proc. Natl. Acad. Sci. USA 87:3062-3066; and Frederikson, et al. (1988) Neuron, 1:439-448). Oncogenes generally used to establish cells from the developing nervous system include v-*myc*, SV40 T antigen, and *neu* (Frederiksen et al., (1988) Neuron, 1:439-448; Gao and Hatten (1994)Development, 120:1059-1070). The cell lines can be identified by genotypic alterations including the expression of exogenous genes such as *lacZ* (Snyder et al., (1992) supra). Transformed cell lines can be established from embryonic cells at various stages of development. Snyder et al. established their v-*myc*-immortalized cells from embryonic day 13 external germinal layer derived cerebellar cells (Snyder et al., (1992) supra).

Others have used embryonal carcinoma (EC) cells to study neural differentiation since EC cells can be obtained in large amounts and can differentiate into cells of various phenotypes (Edde, B. and Darmon M., "Neural Differentiation of Pluripotent Embryonal Carcinoma Cells," Cell Culture in the Neurosciences, Bottenstein and Sato, eds., 273-285 [1985]).

Viral transfection and oncogene transformation can halt the cell differentiation program at specific stages of differentiation (Frederiksen, et al., (1988) Neuron 1:439-448). These methods also result in alteration of the neural precursor cell properties. SV40 large T-antigen oncogene subverted the establishment of granule cell identity (Gao and Hatten, (1994) Development 120:1059-1070). Because of their transformed phenotype and their potential for invasive growth in vivo, these cells are unlikely candidates for cell based therapies (Jiao et al. (1992) Brain Res. 143-147).

Recently, evidence suggests that it should be possible to halt neuroepithelial cell differentiation and allow for infinite proliferation of untransformed murine neural precursor cells in serum free medium supplemented with appropriate growth factors (Loo et al., (1987) Science 236:200-202; Loo et al., (1991) J. Neurosci. Res. 28:101-109). Unlike mouse embryo cells cultured in conventional serum supplemented media these cells do not lose their proliferative potential leading to genetically altered cell lines (Loo et al., (1987)Science, 236:200-202). Researchers have established cell lines from 16 day old embryos which display an absolute requirement for epidermal derived growth factor (EGF) (Loo et al., (1989) J. Cell. Physiol. 139:484-491). An immortal glial precursor cell line (SFME) has been established in the presence of EGF (Loo et al., (1987) supra). Cells have also been isolated from embryonic day 10 mice which have an absolute requirement for fibroblast growth factor (FGF) (Murphy, et al., (1994) J. Neurosci. Res. 25:463-475).

Serum free embryo cells similar to the mouse SFME cells can be derived from embryonic human brain at similar stages of development using EGF (Loo et al., (1991) J. Neurosci. Res. 28:101-109). However, these cell lines, derived from 20-24 week old human embryos ceased proliferation after about 70 population doublings (Loo et al. (1991) supra). EGF responsive cells have also been identified in adult mammalian central nervous system (Reynolds and Weiss Science (1992) 225:1707-1710). EGF was found to support the indefinite growth of subvetricular neural progenitor cells from adult mouse and retinal neural precursor cells (Reynolds and Weis (1992) supra).

It has been demonstrated that basic fibroblast growth factor (bFGF) is a potent mitogen for certain CNS populations (Murphy et al., (1990) supra; Cattaneo and McKay (1990) supra; Ghosh and Greenberg (1995) Neuron 15:89-103; and Vicario-Abejon et al. (1995) Neuron 15:105-114). It also appears that bFGF and nerve growth factor (NGF) cooperatively promote proliferation and differentiation of embryonic striatal neurons (Cattaneo and McKay (1990) supra) However, neither EGF nor FGF support neuroepithelial cells cultured from earlier stages of development as, for example, embryonic day 9 rat neural tube.

Good cellular candidates for cell based therapies such as intracerebral grafting and gene transfer are optimally free of genetic modification and readily available. Additionally, good candidates will exhibit prolonged in vivo survival and non-invasive growth (Gage et al., (1991) TINS 14:328-333). Recently, immortalized neural precursor cells have been used as a carrier for the local delivery of NGF in the brain (Martinez-Serrono (1995) Neuron 15:473-484). The NGF secreting cells expressed a transgene and secreted bioactive nerve growth factor at levels sufficient to reverse cholinergic neuron atrophy in transplanted rats (Martinez-Serrono et al., (1995) supra). These cells were able to survive up to 10 weeks after transplantation and reverse age-dependent cognitive impairments in the model. Another group has transplanted human fetal mesencephalic tissue into the caudate nucleus of patients with Parkinson's disease (Spencer et al. (1992) New England J. Med. 327:1541-1548). Intracerebral grafting has been demonstrated with non-neuronal cell types such as fibroblasts (Gage et al. (1991) Trends Neurosci. 14:328-333; Gage and Fisher, et al., (1991) Neuron 6:1-12; Fisher (1994) Neurochem. Int. 25:47-52) and primary muscle cells (Jiao et al., (1992) 575:143-147) in part to avoid the possibility of tumorigenesis and helper virus contamination that may impact immortalized primary neuronal cells in vivo.

Others have used genetically altered polymer-encapsulated cells to deliver human nerve growth factor transgene product in rat models (Winn et al., (1994) Proc. Natl. Acad. Sci. 91:2324-2328: Hoffman, et al., (1993) Experimental Neurology 122:100-106; Maysinger et al., (1994) Neurochem. Int. 24:495-503). Polymer encapsulation allows diffusion of macromolecules and permits nutrient exposure (Winn et al., (1994) supra).

Snyder et al. have shown that early embryonic precursor cells can engraft and participate in the development of mouse cerebellum (Snyder et al., (1992) Cell 68:33-51)

There is a need therefore for normal neural epithelial cells from early embryonic stage of development that are capable of replication and expansion both in vitro and in vivo for a variety of purposes including cell based therapies.

Summary of the Invention

It has now been discovered that a neural precursor cell type can be isolated from embryonic neural tissue that is capable of continuous in vitro cell culture. The cell type, which is isolated in serum free culture from embryonic neural tissue, does not respond to epidermal growth factor, does not display phenotypic aberration by virtue of viral transfection or oncogene transformation, and can be maintained in continuous culture without undergoing a growth crisis, senescence, or spontaneous differentiation. This cell type has been identified as a neural epithelial precursor cell (NEP cell) expressing the intermediate filament protein nestin. The present invention therefore provides a method for isolating a normal neural epithelial precursor cell type from a developing mammalian embryo.

According to the present invention when neural tissue from the developing mammalian central nervous system at the zero-somite stage of development, equivalent to the rat embryonic day 9, is cultured in the presence of embryonic Schwann cells or embryonic Schwann cell conditioned media, a neural epithelial precursor cell type can be isolated that can be maintained in continuous culture in serum free media. Therefore, the present invention provides a method of isolating a normal neural epithelial precursor cell type comprising the steps of culturing neural tissue from a mammalian embryo at the zero-somite stage of development in the presence of embryonic Schwann cells or embryonic Schwann cell conditioned media and isolating the normal neural epithelial precursor cells.

The invention further provides for the normal neural epithelial cell type isolated according to the methods of the present invention. The neural epithelial cell line of the present invention is of mammalian origin. According to one aspect of the present invention neural tube from a rat embryo at the ninth day of development is cultured according to the methods of the present invention. According to this aspect of the present invention a neural epithelial precursor cell line of rat origin is obtained. In a preferred aspect of the present invention, embryonic tissue from a human embryo at the equivalent stage of development is cultured according to the methods of the invention. According to this aspect of the invention a normal human neural epithelial precursor cell is obtained.

The invention further provides a method for the long term culture, expansion, and maintenance of the cell lines of the present invention. According to the methods of the present invention, the normal neural epithelial precursor cell type can be maintained in long term culture. Preferably the cells are cultured on plates coated with laminin, or extracellular matrix materials containing laminin.

The invention further relates to compositions comprising the normal neural epithelial precursor cells of the present invention. The compositions of the present invention are formulated in a pharmaceutically acceptable vehicle. In one embodiment the phamaceutically acceptable vehicle is a balanced saline solution. In a further embodiment a solid or semisolid, gelatinous, or viscous support medium is the pharmaceutically acceptable vehicle. Preferred gelatinous vehicles include collagen and hydrogels optionally supplemented with the relevant extracellular matrix proteins such as laminin and may include the appropriate growth factors. Such compositions are suitable for transplantation into either the peripheral or central nervous system.

Brief Description of the Drawings

Figure 1A-Figure 1F. Neural epithelial precursor (NEP) cells isolated according to the methods described herein were cultured in the presence and absence of various factors. For Figure 1A cells were grown in F12/DME supplemented with bovine pituitary extract(2 µg/ml), forskolin (5 µM), insulin (10 µg/ml) and transferrin (10 µg/ml) (lane 4F) or in the same media excluding i) bovine pituitary extract (lane BPE), ii) forskolin (lane For), and iii) insulin (lane Ins). Figures 1B-1E describe the results of culturing NEP cells in F12/DME containing bovine pituitary extract (BPE), insulin, heregulin (1 nM), Vitamin E (5 µg/ml), progesterone (3 nM), transferrin (10 µg/ml), and forskolin in wells coated with laminin in the presence of increasing concentrations of forskolin and bovine pituitary extract (Figure 1B), insulin (Figure 1C), insulin like growth factors I and II (Figure 1D), nerve growth factor and epidermal growth factor (Figure 1E). NEP cells in Figure 1F were cultured in F12/DME containing bovine pituitary extract (BPE) (2 µg/ml), insulin (10

μg/ml), heregulin (1 nM), Vitamin E (5 μg/ml), progesterone (3 nM), transferrin (10 μg/ml), and forskolin (5 μM) on laminin coated plates and in the presence or absence of platelet derived growth factor (PDGF), interleukin 1B (IL-1B), interleukin 11 (IL-11), human hepatocyte growth factor (hHGF) and neurotrophin 3 (NT-3).

Figure 2A-Figure 2B. NEP cells were grown in DME/F12 including insulin, transferrin, bovine pituitary extract, forskolin, progesterone, and vitamin E and exposed to the indicated concentrations of heregulin or transforming growth factor ß (Figure 2A) or acidic or basic fibroblast growth factor (Figure 2B).

Detailed Description of the Preferred Embodiments

Definitions

The term "normal" when used in connection with a neural epithelial precursor cell type of the present invention is meant to refer to a cell type which does not display a phenotypic aberration by virtue of viral transfection or oncogene transformation. The normal cell type of the present invention can be maintained in continuous culture, are capable of replication, expansion, and long term culture in vitro while maintaining their ability for differentiation in vitro and in vivo without undergoing a growth crisis, senescence, or spontaneous differentiation. Such cell types are to be distinguished from cell lines that are capable of unlimited proliferative potential and of long term continuous culture only after infection with a transforming vehicle such as adenovirus, SV40, ras, or c-myc. Normal cell lines are non-tumorigenic. By non-tumorigenic is meant that the cell will not give rise to a tumor when injected into syngeneic or immuno-compromised animals.

A "cell line" according to the present invention, is a substantially homogeneous group or population of cells derived through the culture of a sample of cells from a tissue or organ. By "substantially homogeneous" is meant a population of cells that by virtue of common parent or parents are generally phenotypically and genotypically identical. It is to be understood that such uniform cell preparations will deviate as a result of natural mutational changes (allelic variations) that may confer variations in the phenotype in the population of cells. Such natural variations are meant to be included within the definition of substantially homogeneous and therefor are included in the definition of cell line as defined herein. Preferably, "substantially homogeneous" means that between about 90 to 100%, and preferably 99% to 100% of the cells in the population are identical cells.

"Immortalization" is the transformation of a cell culture in vitro into a strain with unlimited growth potential.

"Neural epithelial precursor (NEP) cells" are identified by the expression of the intermediate filament protein nestin (Frederickson and McKay (1988) J. Neurosci. 8:1444-1151; Lendahl, et al., (1990) Cell 60:585-595; Cattaneo and McKay (1990) Nature 347:762-765; Hockfield, S. and McKay R.D.G. (1995) J. Neurosci. 5, 3310-3328). The expression of nestin distinguishes neural epithelial precursor cells from more differentiated cells of neural tube origin (Lendahl, et al, (1990) Cell, 60:585-595; Reynolds and Weiss, (1992) Science, 225:1707-1710). The cells are isolated from neural tissue developmentally equivalent to embryonic day 9 rat neural tube. Neither EGF nor FGF are mitogenic for NEP cells, and they differentiate into a variety of neural cell types in the presence of basic fibroblast growth factor and forskolin.

By "developmentally equivalent" is meant that a particular source of embryonic tissue is obtained from a developing mammalian embryo at an equivalent stage of embryonic development. Embryonic development can be compared between species by the appearance of distinct morphological features. This is distinguishable from gestational time since the timing of appearance of principal morphological features varies between species. According to the present invention, mammalian embryonic tissue is obtained at a stage equivalent to the embryonic zero-somite stage of development. According to the present invention a mammalian embryo is obtained that is developmentally equivalent to a rat embryo at the ninth day of embryonic development. According to the present invention, rat embryonic day nine is measured by mating male and female rats for two hours between the hours of 8 and 10 a.m. and immediately seeking a copulatory plug. The day the copulatory plug is found is defined as day zero of the gestation. For other mammalian species however, it is not the gestational day but the developmental stage of the embryo that is important. Therefore, according to the present invention the rat E9 embryo or the developmentally equivalent embryo from other mammalian species is employed. The rat E9 embryo is generally at the zero-somite stage of development characterized by the appearance of the primitive groove and the allantois, coupled with amnion formation. For developmentally equivalent stages in other mammalian embryos the reader is directed to Kaufman, M. H., "Morphological Stages of Post Implantation Embryonic Development" in Post implantation Mammalian Embryos: A Practical Approach, Copp A., and Cockcroft, D., Eds, Oxford University Press, New York (1990), pp 81-91; Downs and Davies, (1993) 118:1255-1266; and Fujinaga and Baden (1992) Teratology 45:661-670; and O'Rahilly, R. and Müller, F. (1987), "Developmental Stages in Human Embryos," Carnegie Institution of Washington, Publication no. 637. Carnegie Institute, Washington DC.

A "Schwann cell" is a cell of neural crest origin that forms a continuous envelope around each nerve fiber of peripheral nerves in situ. A Schwann cell can be identified as such by detecting the presence of one or more Schwann cell markers such as glial fibrillar acidic protein (GFAP), or S100 protein, *e.g.*, using antibodies against these markers. Furthermore, Schwann cells have a characteristic morphology which can be detected by microscopic examination of cultures thereof. Isolated Schwann cells can also be evaluated for the maintenance of differentiated Schwann cell functions, such as the ability to associate with sensory neurons in culture or the ability to produce myelin or myelin related proteins such as Po and myelin associated glycoprotein (MAG). An "embryonic Schwann cell" is a Schwann cell or Schwann cell precursor isolated from an embryonic mammal at a stage developmentally equivalent to the rat embryonic day 14 up to birth. Preferably, the embryonic Schwann cell is isolated from a mammal at a stage developmentally equivalent to rat embryonic day 14 through rat embryonic day 18, and most preferably at about embryonic day 15.

The terms "cell culture medium" and "culture medium" refer to a nutrient solution used for growing mammalian cells that typically provides at least one component from one or more of the following categories:

1) an energy source, usually in the form of a carbohydrate such as glucose;
2) all essential amino acids, and usually the basic set of twenty amino acids plus cystine;
3) vitamins and/or other organic compounds required at low concentrations;
4) free fatty acids; and

5) trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range.

The nutrient solution may optionally be supplemented as described herein with one or more components from any of the following categories:

1) one or more mitogenic agents;

2) salts and buffers such as, for example, calcium, magnesium, and phosphate;

3) nucleosides and bases such as, for example, adenosine and thymidine, hypoxanthine; and

4) protein and tissue hydrolysates.

According to the present invention the cell culture medium is generally "serum free", which means that the medium is essentially free of serum from any mammalian source (*e.g.* fetal bovine serum [FBS]). By "essentially free" is meant that the cell culture medium comprises between about 0-5% serum, preferably between about 0-1% serum and most preferably between about 0-0.1% serum (v/v).

A "mitogenic agent" or "growth factor" is a molecule which stimulates mitosis of the particular cell type under investigation. Generally, the mitogenic agent or growth factor enhances survival and/or proliferation of the cell type in cell culture. Examples of mitogenic agents include Rse/Axl receptor activators; activators of one or more members of the *erb*B receptor family; agents which elevate cAMP levels in the culture medium (*e.g.* forskolin, cholera toxin, cAMP or analogues thereof); adhesion molecules such as neural cell adhesion molecule (N-CAM), laminin or fibronection; progesterone; neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and ciliary neuronotrophic factor (CNTF); neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6); or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); epidermal growth factor (EGF); fibroblast growth factor such as acidic FGF (aFGF) and basic FGF (bFGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factors, including IGF-I, IGF-II and des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins such as IGFBP-1, -2, -3, -4, -5, or -6; and hormones such as estrogen, testosterone, progesterone, thyroid hormone, and insulin.

"Pharmaceutically acceptable" carriers or vehicles are ones which are nontoxic to the mammal being exposed thereto at the dosages and concentrations employed. Often the pharmaceutically acceptable carrier is an aqueous pH buffered solution. Examples of pharmaceutically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG). Pharmaceutically acceptable carriers or vehicles also include semi-solid, gelatinous or viscous support medium. Examples of such gelatinous carriers or vehicles include collagen, collagen-glycosaminoglycan, fibrin, polyvinyl chloride, polyamino acids such as p lylysine or polyornithine, hydrogels, agarose, dextran sulfate

and silicone. Pharmaceutically acceptable vehicles appropriate for the transplantation of developing nerve cells are found in, for example International Publication No. WO 92/03536.

A nervous system disease or disorder is meant to include traumatic lesions (*e.g.* caused by physical injury or surgery, and compression injuries); ischemic lesions (*e.g.* cerebral or spinal cord infarction and ischemia); malignant lesions; infectious lesions (*e.g.* resulting from an abscess or associated with infection by human immunodeficiency virus, Lyme disease, tuberculosis, syphilis, or herpes infection); degenerative lesions (*e.g.* associated with Parkinson's disease, Alzheimer's disease, Huntington's chorea or amyotrophic lateral sclerosis); lesions associated with nutritional diseases or disorders (*e.g.* Vitamin B12 deficiency, folic acid deficiency, Wernicke disease, tobacco-alcohol amblyopia, Marchiafava-Bignami disease and alcoholic cerebellar degeneration); neurological lesions associated with systemic diseases (*e.g.* associated with diabetes, systemic lupus erythematosus, carcinoma or sarcoidosis); lesions caused by toxic substances (*e.g.* alcohol, lead or neurotoxin); and demyelinated lesions (*e.g.* associated with multiple sclerosis, human immunodeficiency virus-associated myelopathy, transverse myelopathy of various etiologies, progressive multifocal leukoencephalopathy and central pontine myelinolysis).

The term "treatment" within the instant invention is meant to include therapeutic treatment, as well as prophylactic, or suppressive measures for a disease or disorder. Thus, for example, in the case of Alzheimer's disease, successful administration of an agent prior to onset or during the course of the disease results in "treatment" of the disease whether or not associated with delayed or prevented onset of the disease if the administration is associated with an apparent clinical benefit. Such a benefit may be any benefit including but not limited to the lessening of a side effect of another therapy or the disappearance of a symptom. For example, successful administration of the agent after clinical manifestation of the disease to combat the symptoms of the disease comprises "treatment" of the disease. "Treatment" also encompasses administration of the agent after the appearance of the disease in order to eradicate the disease. Successful administration of the agent after onset and after clinical symptoms have developed, with possible abatement of clinical symptoms and perhaps amelioration of the disease, also comprises "treatment" of the disease.

The term "mammal" refers to any mammal classified as a mammal, including humans, non-human primates, cows, horses, dogs, sheep and cats. In a preferred embodiment of the invention, the mammal is a human.

Modes for Carrying out the Invention

It will be recognized by the skilled artisan that for the in vitro culture of the neural epithelial precursor cells of the present invention routine aseptic cell culture techniques are to be employed along with the methods of the present invention. The invention will now be described with reference to these and other cell culture techniques.

For the isolation of the normal neural epithelial precursor cells (NEP cells) of the present invention mammalian neural tissue is obtained from a mammal in the zero-somite (0-somite) stage of development. This stage of the developing embryo is characterized by the appearance of the neural groove, the developing allantois and amnion formation. The stage precedes the appearance of the first s mites and is generally identified by the appearance of the neural plate. According to the present invention, this stage is

developmentally equivalent to the ninth day of rat embryonic development. Therefore, according to the present invention embryonic day nine rat neural tissue or developmentally equivalent neural tissue in other mammalian species is employed. According to a preferred aspect of the invention, embryonic neural tissue at the ninth day of rat embryonic development between the hours of 3 p.m. and 4 p.m. or developmentally equivalent neural tissue from another mammalian species is employed.

One skilled in the art will recognize that mammalian embryos follow similar developmental patterns that can be identified as distinct morphological stages. The stages are identified broadly by day of appearance of the particular morphological feature, such as the neural plate. The timing of appearance of principle morphological features, however, varies between species. Accordingly, embryonic neural tissue is isolated just prior to the appearance of the first somites. This stage is commonly referred to as the "zero-somite" or "0-somite" stage of development. In the rat species the first somites appear between the 9th and 10th day of embryonic development and developing neural tissue is obtained at the ninth day of development just prior to the appearance of the first somites. The rat species anticipates the murine species developmentally by 1.5 to 2 days for the appearance of the same principal features. Accordingly, in the mouse species, the first somites appear at about the eighth day of embryonic development. Therefore, for the murine species, neural tissue is generally obtained at about between the sixth to about the eighth day of murine embryonic development. In the human, the first somites appear during the ninth stage of human development at about between the twentieth and twenty-fourth day of embryonic development. Therefore according to the present invention human mammalian neural tissue is generally selected from the developing human embryo after about the eighteenth to twenty-eighth day of embryonic development.

Neural tissue obtained from the embryo developmentally equivalent to the E9 rat embryo is generally isolated from the developing embryo by dissection employing techniques commonly available to the skilled artisan. One skilled in the art of mammalian developmental biology will be able to identify and isolate the neural tissue using techniques that are standard in the art. Generally microscopic dissection is employed to separate the caudal portion of the embryo, followed by removal of the developing mesoderm and endoderm. According to the present invention the remaining neural tissue is employed and preferably the neural tube is employed.

Neural tissue and preferably neural tube from the developing embryo at rat E9 or the developmentally equivalent stage is cultured in the presence of embryonic Schwann cells or advantageously in the presence of culture media obtained from the in vitro culture of embryonic Schwann cells. Embryonic Schwann cells are isolated by the methods described herein or by methods known to those skilled in the art. The embryonic Schwann cells may be a primary or secondary culture or a continuously propagated line as described herein. Additionally, continuous embryonic Schwann cell lines obtained by virtue of viral transformation or oncogene transfection may be employed as a source of embryonic Schwann cells or embryonic Schwann cell conditioned media. Alternatively, the embryonic Schwann cells may be a Schwannoma as described by (Ansselin and Corbeil (1995) In Vitro Cell Dev. Biol. 31:253-254; Brockes et al. (1979) Brain Res. 277:389-392; Morrissey et al. (1991) J. Neurosci. 11(8):2433-2442; Peulve et al. (1994) Exp. Cell. Res. 214:543-550; Rutkowski et al. (1992) Ann. Neurol. 31:580-586; Watabe et al. (1990) J. Neuropathol. Exp. Neurol. 49:455-467) for example. Embryonic Schwann cells and methods of obtaining them are known to those skilled in the art

(Messing et al. (1994) J. Neurosci. 14:3533-3539). Copending U.S. Application Serial No. 08/435,436 filed May 10, 1995 describes an exemplary method of obtaining embryonic Schwann cell cultures.

In obtaining Schwann cells or in selecting nerve tissue which comprises Schwann cells (*e.g.* peripheral nerve tissue) for isolation of the Schwann cells it is important that the cells be derived from an embryonic mammal. According to the present invention, an embryonic Schwann cell is obtained from a mammal at a stage developmentally equivalent to rat day 14 stage of embryonic development or at any stage thereafter through the last day of embryonic development. Preferably, Schwann cells developmentally equivalent to rat embryonic day 15 are employed. Appropriate tissue sources include, for example, dorsal root ganglia.

In a preferred aspect of the present invention, the mammalian species from which the Schwann cells are derived matches the species from which the normal NEP cells are isolated. However, species matching is not an absolute requirement. For example, rat embryonic Schwann cells may be employed as the embryonic Schwann cell source for the isolation of murine as well as human normal NEP cells in addition to rat normal NEP cells.

In an exemplary method of isolating embryonic Schwann cells, nervous tissue from a developing embryo (generally between rat day 14 and day 20) is obtained. A suitable size for the starting nerve tissue is generally between about 1mg to 1gm. The nerve tissue can be stored in medium (*e.g.* RPMI-1640 [Sigma], Liebovitz's L15 or Belzer's UW solution), prior to culture. Pre-incubation of the embryonic Schwann cell may be advantageous if the tissue contains myelinated nerves to facilitate demyelination of the Schwann cells. For the pre-incubation of embryonic Schwann cells, the nerve tissue is desirably treated with one or more protease enzymes for a sufficient period of time to loosen connective tissue and thereby promote demyelination. Many protease enzymes are commercially available which can be used for this step and include collagenase, dispase and other serine proteases, for example. Excess enzyme can be removed by gentle washing with culture medium.

Embryonic Schwann cell culture is typically carried out on a solid phase (*e.g.* a plastic tissue culture dish or plate) coated with extracellular matrix/adhesion proteins such as laminin, fibronectin, poly-lysine or collagen, with laminin being preferred. This allows preferential adhesion and migration of the Schwann cells onto the coated solid phase. The Schwann cells are cultured in the laminin-coated culture plates in a suitable culture medium.

Culture media for the culture of embryonic Schwann cells are well known to persons skilled in the art and include, but are not limited to, commercially available media such as F12/ DME, Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma). Any of these media may be supplemented as necessary with, ions (such as sodium, chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Other supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The preferred culture medium is F12/DME (1:1) containing 4.5 g/liter glucose supplemented with 15 mM HEPES, pH 7.4, 1.2 g/liter sodium bicarbonate.

According to the present invention, whereas the medium may be supplemented as described above to promote the growth and expansion of the embryonic Schwann cells in vitro, for the purposes of the present invention, serum free culture conditions are employed. The embryonic Schwann cells are preferably placed in dishes (*e.g.* 100mm petri dishes) in culture medium supplemented with suitable mitogenic agents. For example, for the rat embryonic Schwann cell the culture medium is preferably serum free medium which is supplemented with one or more mitogenic agents including, at least an *erb*B activator (*e.g.* heregulin), insulin (or an IGF), bovine pituitary extract (Roberts et al., (1990) Am. J. Physiol. 3:415-425), and a stimulator of cAMP production (e.g. forskolin) in the appropriate ranges to promote growth and expansion of the embryonic Schwann cells and avoiding the use of growth factors such as acidic or basic fibroblast growth factor, epidermal growth factor, and the like.

Therefore, in an exemplary method of isolating rat embryonic Schwann cells for co-culture according to the present invention dorsal root ganglia from E14 rat embryos or the developmentally equivalent embryo from other mammalian sources is isolated. Clean dorsal root ganglia are incubated with collagenase/dispase (Boehringer Mannheim Cat. No. 1097113) at a concentration of 0.3% for about 45 minutes. The dorsal root ganglia is then rinsed clean and dispersed by gentle pipetting with a 1 ml Pipetman™. The dispersed cells are collected by centrifugation (1000 rpm, 5 min) washed three times with F12/DMEM and plated on laminin coated plates in F12/DMEM supplemented with recombinant human insulin (5µg/ml), transferrin (10µg/ml), progesterone (2×10^{-8} M), bovine pituitary extract (Roberts et al. supra)(10µg/ml), recombinant heregulin (3 nM, HRG-ß1$_{177-244}$) (Holmes et al. [1992] Science 256:1205-1210), forskolin (5 µM) and α-tocopherol (5 µg/ml). Schwann cells grow to a confluent monolayer after 4 days.

Schwann cells from the primary cultures grown on the laminin substrate are passaged every 4 days at a 1:4 split ratio onto laminin-coated dishes in medium supplemented with progesterone, insulin, α-tocopherol, heregulin, forskolin, and transferrin, as well as bovine pituitary extract. Culture media from the culture of embryonic Schwann cells is obtained from embryonic Schwann cell cultures at any time after the cells have reached confluency. Preferably Schwann cell conditioned media is collected after about 2-4 days and most preferably after about 4 days of culture.

For the isolation of embryonic neural epithelial precursor cells by co-culture with embryonic Schwann cells or embryonic Schwann cell conditioned medium, dispersed neural tissue obtained as described above from the rat E9 embryo or the developmentally equivalent mammalian source is placed in culture with embryonic Schwann cells (ESC's) or in culture medium supplemented with embryonic Schwann cell conditioned medium (ESCCM). Co-culture of the neural tissue with the ESC's may occur by virtue of the cells being placed together in the same culture vessel, as well as by co-culture where the cells, i.e., ECS's and the cells of the developing neural tube, are separated by a physical barrier which allows the transport of soluble factors across a semipermeable membrane while avoiding physical contact of the cells themselves.

According to the present invention an appropriate culture vessel is previously coated with a suitable extracellular matrix protein such as laminin. Appropriate culture vessels for the isolation and culture of neural epithelial precursor cells of the present invention include glass and plastic vessels. Although glass vessels may be employed, generally the solid phase is a plastic or polystyrene tissue culture dish or plate such as those routinely employed for mammalian cell culture. The tissue culture vessel is selected keeping in mind that a

small quantity of cells is initially cultured. Generally, between about 100 and 1 X 10^5 are plated onto or into the vessel. The vessel is selected so that the cells remain in contact in a small, generally about 100 to 200 microliter volume. Appropriate tissue culture vessels include plastic 96 well plates.

In addition the plastic plate or tissue culture vessel is normally coated with an extracellular matrix or attachment factor that contains some or all of the components of the extracellular matrix that most cells of the developing vertebrate organism are in contact with during normal development. Collagen and laminin are generally produced by the developing mammalian neuroblast. Therefore, extracellular matrix/adhesion proteins such as collagens, glycosaminoglycans, proteoglycans, and glycoproteins are employed. Most preferred are the glycoproteins such as fibronectin, laminin, enatactin, and hyaluronectin. Most preferred according to the present invention is laminin.

The medium used in the co-culture isolation of the neural epithelial cells of the present invention may be any of a number of synthetic basal medium that are commercially available. The medium is selected bearing in mind that basal media differ in their survival, growth, and differentiation promoting properties. Suitable culture media include, but are not limited to, commercially available media such as F12/ DME, Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma). In addition, any of the media described in Ham and Wallace, Meth. Enz., 58:44 (1979), Barnes and Sato, Anal. Biochem., 102:255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985; or U.S. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as a basal culture medium. The preferred culture medium is F12/DME (1:1).

These media and especially F12/DME may be supplemented with ions (such as sodium, chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source.

According to the present invention, for the isolation of neural epithelial precursor cells from co-culture with embryonic Schwann cells or embryonic Schwann cell conditioned medium, the basal medium described above is additionally supplemented with bovine pituitary extract (Roberts et al., (1990) Am. J. Physiol. 3:415-425) at about 0.01 to about 100 µl/ml and preferably at about 3-5 µl/ml. An agent which elevates cAMP levels in the medium is additionally desirably present. Forskolin is the preferred such agent and suitable concentrations of this molecule are in the range from 0.1 µM to 50 µM forskolin, more preferably 1µM to 20µM forskolin, and most preferably about 5 µM forskolin. Insulin or an IGF (*e.g.* IGF-I or IGF-II) is also desirable for the co-culture. Preferably, insulin will be added to the medium in the range 0.01 µg/ml to 100µg/ml, more preferably 1µg/ml to 10µg/ml, and most preferably 10µg/ml. Additionally, the culture media may optionally include a mitogenic agent such as a molecule which activates a member of the *erb*B receptor family. Heregulin is the preferred activator and the human heregulin-$\beta 1_{177\text{-}244}$ fragment is the most preferred mitogenic agent (Holmes et al., (1990) Science, 256:1205-1210). The concentration of heregulin in the medium will preferably be in the range of about 0.001nM to 10nM, more preferably 0.1nM to 10nM, and most preferably 1nM to 10nM. Progesterone may also be added to the culture medium in the range of about 0.1nM to 200nM, and more preferably about 1nM to 100nM, and most preferably about 3nM. An iron source such

as transferrin may be employed. The concentration of transferrin in the medium will generally be in the range of about 0.1 μg/ml to about 100 μg/ml and preferably between about 5 μg/ml and 10 μg/ml. Other optional supplements include Vitamin E (an anti-oxidant and anti-transforming agent), preferably in the range 0.1 μg/ml to 100 μg/ml, more preferably 1 μg/ml to 20 μg/ml, and most preferably 5 μg/ml to 10 μg/ml; and chemically defined lipids (Sigma Cat # 11905-015), preferably in the range from about 1μL/ml to 500μL/ml, more preferably 10μL/ml to 100μL/ml, and most preferably 25μL/ml to 50μL/ml.

A preferred media formulation according to the present invention includes recombinant human insulin at about 5μg/ml, progesterone at about $2x10^{-8}$ M, bovine pituitary extract (Roberts et al., (1990) Am. J. Physiol. 3:415-425) at about 10μg/ml, recombinant heregulin at about 3 nM (HRG-ß$1_{177-244}$ (Holmes et al. [1992] Science 256:1205-1210), forskolin at about 5 μM, transferrin at about 5 μg/ml and α-tocopherol at about 5 μg/ml.

Culture conditions, such as temperature, pH, dissolved oxygen (dO_2) and the like, are those generally used in mammalian cell culture and will be apparent to the ordinarily skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells is between about 30 to 38°C and a suitable dO_2 is between 5-90% of air saturation.

Advantageously, the cells of the present invention may be co-cultured and isolated with embryonic Schwann cell conditioned medium in place of the embryonic Schwann cells. According to this aspect of the present invention, the media described above is supplemented with culture media obtained from the culture of embryonic Schwann cells. Preferably, such conditioned media is obtained from the culture of embryonic Schwann cells after about 1 to 4 days post-initiation of an embryonic Schwann cell culture. Minimally, the embryonic Schwann cells should have reached confluency prior to harvesting the culture media. Preferably, the culture supernatant from the embryonic Schwann cells is harvested by removal of the cells. This is generally accomplished by centrifugation and/or filtration. The remaining culture media is used as a media supplement.

Embryonic Schwann cell conditioned media obtained in this manner can be used as a media supplement as obtained, or it may first be concentrated. Concentration is generally accomplished keeping in mind that the concentration devise should allow for the retention of essentially all proteinaceous substances. This generally means using a device with a low protein binding molecular weight cutoff of about between 3000 and 5000 kilodaltons. Concentration advantageously allows for smaller volume additions to the media described.

The amount of embryonic Schwann cell conditioned media (ESCCM) employed as a media supplement is not a fixed amount but varies with the batch of conditioned media employed. The ESCCM should be present in at least an amount sufficient to promote the growth of the neural epithelial cells of the present invention. Embryonic Schwann cell conditioned media is added at a ratio of about 1:1 to about 10:1 parts of the media formulation described above to the embryonic Schwann cell conditioned media and preferably at about 5 parts of the media formulation described above to about 1 part ESCCM. With ESCCM concentrated prior to addition as a media supplement the amount of ESCCM media added as a supplement

varies according to the concentration. For ESCCM concentrated 10 fold, the ESCCM is added to the basal media in the range of 1% to about 50% (v/v) and preferably about 30% (v/v).

When the neural tissue is placed in co-culture with the embryonic Schwann cells it is not necessary to supplement the basal media with embryonic Schwann cell conditioned medium. Neural tissue is plated onto plates precoated with the appropriate extracellular matrix to which embryonic Schwann cells have been added. The number of embryonic Schwann cells used in co-culture varies with the size of the culture vessel. An amount of embryonic Schwann cells sufficient to form a monolayer in the culture vessel is sufficient. Generally embryonic Schwann cells plated at an initial density of about 1 to about 5 x 10^5 cells per cm^2 culture medium is sufficient.

Neural epithelial precursor cells obtained from the dispersed neural tissue as described above are plated keeping in mind that the cells require cell to cell contact in order to survive. Optimal cell growth is achieved at an initial plating density of about between 0.5 to about 5 x 10^5 cells per ml.

Under these conditions neural epithelial precursor cells survive and proliferate to form large colonies of compacted monolayer epithelial cells containing some differentiated neurons bearing long processes.

After about 2-6 days in culture, and generally after about 4 days in culture, colonies of neural epithelial precursor cells obtained as described above are removed from the extracellular matrix protein coated plates and replated in the media as described above at a density of about 1-2 x 10^5 cells/ml. The cells are subcultured by repeating this process for several cell passages. Generally about 5 to 6 passages are performed.

During this period the cultures contain two major cell types, one is the neural epithelial precursor cell type of the present invention and the other is a bipolar Schwann cell-like cell. The bipolar Schwann cells are removed from the culture by removing heregulin from the culture medium.

Following this, normal neural precursor cell lines are maintained in culture in media as described and preferably in F12/DME supplemented with forskolin, BPE, transferrin, progesterone, and α-tocopherol. Embryonic Schwann cells or embryonic Schwann cell conditioned media is generally only required to isolate the cell line of the present invention.

Cell lines isolated from embryos can be frozen for long term storage in serum free media containing 10% dimethylsulfoxide (DMSO) or glycerol.

USE

Neural epithelial precursor cells produce a number of neurotrophic and neural growth-promoting factors (Placzek et al. (1993) Development 117:205-218; Ghosh and Greenberg (1995) Neuron 15:89-103)and themselves respond to a number of growth promoting substances. Accordingly, normal neural epithelial cells isolated and cultured using the techniques described herein can be used to produce and detect these factors. It is desirable to have populations of normal neural epithelial precursor cells in cell culture for isolation and detection of neural epithelial precursor cell specific factors such as sonic hedgehog (SHH) (Hynes, M. et al.,(1994) Neuron, 15:35-44). Such factors are useful as diagnostic tools themselves or can be used an antigens to generate antibodies for diagnostic use.

The cell lines themselves are useful for diagnostic purposes. For example, the cells of the present invention may be used to detect the presence of various growth promoting substances. According to this aspect

of the present invention, the cells may be used to monitor the isolation of specific neural epithelial growth promoting factors during the course of an isolation procedure. Cell based assays can be simply devised utilizing the neural epithelial precursor cells of the present invention. For example, the synthesis of DNA and the incorporation of a radiolabelled nucleotide are commonly employed in cell based assays for determining the responsiveness to a particular sample containing a growth promoting substance. Such cellular assays may be diagnostically relevant for the detection of the presence or absence of clinically relevant substances or in the course of a therapeutic treatment.

Alternatively, the normal neural epithelial cells may be used for the recombinant expression and production of various proteins. According to this aspect of the invention the cell lines isolated by means of the present invention may be transfected with an expression vector containing a nucleic acid sequence encoding a protein of choice. One of ordinary skill in the art will choose an appropriate DNA expression system for use in association with the cell lines of the present invention. Proteins produced in this manner advantageously possess the particular glycosylation patterns characteristic of the neural epithelial precursor cell.

It is also beneficial to have populations of mammalian NEP cells (preferably human NEP cells) for use in cell based therapies. The NEP cells of the present invention may be used alone or in combination with other cell types for direct transplantation into areas of damaged neural tissue for the treatment of diseases or disorders of the nervous system. The cells alone or in combination with other cell types provide direct replacement and auxiliary support for the regeneration of or repopulation of a nervous system lesion. According to this aspect of the invention the normal neural epithelial cells are formulated in an appropriate pharmaceutical vehicle as described herein. Isolated NEP cells, pharmaceutical compositions comprising NEP cells, or prostheses filled with the NEP cells can be introduced into a nervous system lesion using any method known in the art for the treatment of neurological diseases such as those mentioned above and especially, neurodegenerative diseases or disorders including degenerative lesions associated with Parkinson's disease, Alzheimer's disease, Huntington's chorea or amyotrophic lateral sclerosis.

In a particular embodiment the normal neural epithelial cells of the present invention may be used to repopulate areas of neuronal damage or degeneration. This type of cell based therapy is analogous to the unilateral transplantation of human fetal mesencephalic tissue into the caudate nucleus of patients with Parkinson's disease (Spencer et al., (1992) New Engld. J. Med. 327:1541-1548). Alzeheimer's disease and other disorders such as Down's syndrome, post encephalitic Parkinsonism, dementia pugistica, are characterized by neuronal cell loss and changes in neuronal morphology (Goldman, and Côté (199) "Aging of the Brain"). The neural epithelial precursor cells of the present invention which are capable of in vivo differentiation and integration can be used to repopulate specific areas of damaged neuronal tissue. Methods for delivering cells to the brain are generally known and described in International Publication No. WO 90/06757 and WO 93/14790.

Neural transplantation or "grafting" involves transplantation of the neural epithelial precursor cells into or onto the central nervous system of a host or recipient. These procedures include the transplantation of the neural epithelial precursor cells within a host brain, such as intraparenchymal transplantation, as well as the deposition of the cells on the surface of the host brain in a gelatinous vehicle such as those described below. Intraparenchymal transplantation can be accomplished by injecting the neural epithelial precursor cells of the

invention into a host or recipient brain parenchyma. Procedures for the transplantation of cells into selected regions of a host brain are generally known and include those described in International Publication No. WO 90/06757 as well as the references cited therein.

For grafting, generally, the isolated neural epithelial precursor cells are suspended in a pharmaceutically acceptable vehicle such a balanced glucose-saline solution and injected into a predetermined region of the host brain using a sterile microsyringe. Therefore, the present invention includes a method of transplanting cells comprising isolating a normal neural epithelial cell type, and injecting or grafting the normal neural epithelial cell into or onto a recipient brain.

In a particular embodiment, the normal neural epithelial cells may be used to produce neural epithelial precursor cell specific proteins for delivery of endogenous neurotrophic and neural growth promoting factors in vivo. Alternatively, a variety of methods are currently available to genetically modify cells of neural origin for site specific delivery of biologically active proteins. For example, in Alzheimer's disease, a deficiency of acetylcholine can be corrected by delivering the acetylcholine using the present cell line as a vehicle for the in situ expression of the neurotransmitter. Recently, neural precursor cells have been used as a carrier for nerve growth factor in the brain (Martinez-Serrono (1995) Neuron 15:473-484). For example, it may be possible to introduce a gene encoding a specific molecule which is known to promote nerve fiber growth, or a gene for a specific growth factor molecule believed to sustain or promote neuronal health. The neural epithelial precursor cells may be transfected or infected with a DNA sequence encoding a biologically active protein such as mammalian nerve growth factor or neurotransmitter and subsequently used for increased localized production of the protein. Cell and site specific expression of specific molecules will generally result in local production of prophylactically and therapeutically effective amounts of the desired biologically active protein. Methods for transplanting cells which may or may not express a transfected gene product in the brain are provided generally in International Publication No. WO 93/14790 and WO 93/10234.

By way of example and not of limitation, a recombinant retroviral vector comprising a DNA sequence encoding a biologically relevant protein is utilized to directly infect the neural epithelial precursor cells of the present invention in vitro. The infected cells can then be delivered to the specific tissue target site utilizing methods known in the art including but not limited to techniques which are commonly employed to repopulate areas of suspected neuronal damage. Any number of retroviral constructs which express a biologically active protein may be utilized by the skilled artisan according to this aspect of the invention.

As used herein, the term "pharmaceutically acceptable" generally means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Pharmaceutical formulations comprising isolated NEP cells and a pharmaceutically acceptable carrier or vehicle as described herein can be prepared using techniques which are well known in the art. Suitable formulations include biological buffers, such as phosphate buffered saline, saline, Dulbecco's Media, and the like. The formulation of choice can be accomplished using a variety of the aforementioned buffers, or even excipients including, for example, pharmaceutical grades of glucose, mannitol, lactose, starch, magnesium stearate, sodium saccharin cellulose, magnesium carbonate, and the like. Optionally, the pharmaceutical formulation may include one or more mitogenic agents and other components (*e.g.* extracellular matrix proteins such as laminin). If a gelatinous

support is used as the pharmaceutically acceptable vehicle, the NEP cells may be introduced into a liquid phase of the vehicle which can be treated such that it becomes more solid (*e.g.* a unpolymerized vehicle may be induced to polymerize). For example, the NEP cells may be added after collagen solubilized in acetic acid in H_2O is brought to neutral pH, by addition of a suitable base such as NaOH and to isotonicity by the addition of salts.

The NEP cells can also be delivered in prostheses or devices such as those which have been described in the literature. Generally, a solid porous tube filled with the NEP cells (preferably formulated in a gelatinous vehicle) is used as the prosthesis.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLES

EXAMPLE I

Introduction

Given their developmental potential, neuroepithelial cells from embryonic day 9, 0-somite rat embryos were grown in a serum free hormone supplemented medium. Here the isolation, establishment and characterization of a neuroepithelial precursor cell line which can be induced to differentiate into neural cell types in vitro and in vivo is demonstrated.

Methods

A. Neuroepithelial cell growth:

E9 rat neural tubes (0-somite) were dissected under a dissecting microscope using fine needles. The caudal portion was removed by cutting through the middle of Hassen's node. Mesoderm and endoderm were removed after a brief incubation with collagenase and dispase (Boehringer Mannheim, 0.2%, 10 min at 0 C). The clean neural tubes were washed 5x by transfer from plate to plate of F12/DMEM with 1% BA. The neural tubes were then dispersed into small aggregates (preferentially 20 to 50 cells in each aggregate) by gentle pipetting with yellow tips on a 200μl Pipetman™ pipetter. Complete dispersion should be avoided. The dispersed cells were plated in 96 well multiplates (at a density of 20 neural tubes divided into 96 wells) precoated with attachment factors in serum free medium supplemented with different combinations of growth factors. Good cell survival and growth was found only in conditions which contained bovine pituitary extract (Roberts et al 1989 supra), insulin, forskolin and either in co-culture with the embryonic Schwann cell line or with concentrated Schwann cell conditioned medium (ESCCM). In these conditions, some precursor cells survived and proliferated to form large colonies of compacted monolayer epithelial cells containing some differentiated neurons bearing long processes. Cells in other conditions died within 4 days in culture.

B. Long term culture

Colonies of epithelial cells formed in the primary culture in F12/DME supplemented with 7F (forskolin (about 5 µM), BPE (about 10 µg/ml), insulin (about 5 µg/ml), transferrin (about 5 µg/ml), heregulin (about 3 nM), progesterone (about 2 x 10^{-8}M), α-tocopherol (about 5µg/ml)) and ESCCM, were removed from

the substrate with 0.2% collagenase/dispase at 37° C. The cells were washed free of the enzymes by centrifugation on a layer of 3% BA and plated onto laminin coated 24 well plates with F12 / DME supplemented with 7F. The cells were routinely subcultured by the same procedure for 5-6 passages. During this period the cultures contain two major cell types, one is the compact epithelial cells, the other is bipolar cells resembling Schwann cells or radial glia which were subsequently removed from the culture by removing heregulin from the culture medium and allowing the epithelial cells to grow at a higher density. The cells were then carried in 6F medium (7F medium without heregulin) on laminin coated plates and passaged every week using a 1 to 4 split.

C. Cell proliferation studies

Cells between passages 25 and 35 were used in the experiments described below. Cells were routinely detached from the culture vessels and plated at the density indicated in laminin coated 24 well coated plates in F12/DME medium with different concentration of the growth factor to be tested. Alternatively the cells were plated in 6F for 24 hours and then the growth factor being studied was added at different concentrations in the presence of all other growth factors in 6F. Cell numbers were counted with Coulter counter after complete dispersion of the cells with trypsin-EDTA (Gibco BRL) at 37° C at least for 15 min.

D. Cell differentiation studies:

Cells were plated in 6F (Insulin (5 μg/ml), transferrin (5μg/ml), α-tocopherol (5 μg/ml), progesterone (2×10^{-8}m), forskolin (5μM), and bovine pituitary extract (10 μg/ml)) at 1:8 split in laminin coated 24 well plates with F12/DME supplemented with 6F for 48 hours and then washed with F12/DME and refed with fresh medium supplemented with insulin, transferrin and α-tocopherol. Growth factors such as bFGF (recombinant human bFGF, Gibco BRL), aFGF (recombinant human aFGF, Gibco BRL), forskolin (Calbiochem), and/or NGF(7.5 S mouse NGF, Collaborative Research Inc.) were added individually or in combinations, as indicated. Medium was changed once after a 48 hour incubation with the respective factors. Cultures were fixed for immunocytochemistry at the indicated time with ice cold 1% glutaraldehyde (Ted Pella, Inc.) in a 3% sucrose solution on ice for 30 minutes. The fixed cells were rinsed with ice cold milli-Q water, treated with methanol containing 3% H_2O_2 for 30 minutes at room temperature and washed with PBS. The cells were then incubated with PBS containing 0.2% TRITON X-100™ detergent, 5% goat serum and 0.1 M NH_4HCO_3 for 1 hour at 37° C. Primary antibodies were diluted in dilution buffer (PBS with 0.1% TRITON X-100™ detergent and 1% BA) and were added at 250 µl/well. Incubation with primary antibodies was carried out at 4° C overnight. Excess primary antibody was washed 5x with the dilution buffer and the cells were then incubated with appropriate enzyme conjugated secondary antibodies (Anti-mouse Ig F(ab)'-alkalinephosphatase, Anti-mouse Ig F(ab)'-peroxidase and anti-rabbit IgG F(ab)'-peroxidase were purchased from Boehringer Mannheim) at 37° C for 60 min. The cells were washed 5x with dilution buffer and 2x with either 0.05 M sodium acetate pH 5.0 for peroxidase conjugates or with phosphatase substrate buffer. Specifically bound peroxidase activity was detected with DAB-H_2O_2 solution made using the Sigma peroxidase substrate kit. Alkaline phosphatase was detected with Boehringer alkalinephosphatase substrate (NTB/BCIP) diluted in substrate buffer. The color developing reaction was stopped by thoroughly rinsing with tap water and the specimens were preserve in

glycerol/PBS (50:50). The stained samples were observed and micrographs were taken using a Nikon-overted bright field microscope.

E. Immunofluorescence

For nestin immunofluorescence, cells grown on chamber slides were fixed in situ with 4% paraformaldehyde in phosphate buffer PH 7. for 30 minutes at room temperature. The fixed cells were washed 3x with PBS. blocked with 5% goat serum in PBS plus -0.2% TRITON X-100™ detergent at 37° C for 60 minutes. Anti-nestin antiserum raised in rabbit against the last 1200 amino acid in the rat nestin sequence. The antiserum was diluted at 1:1000 with dilution buffer (and incubated with the cells at 4° C overnight). After 5 washes with dilution buffer (PBS with 0.1% TRITON X-100™ detergent and 1% BA), the samples were incubated with goat anti-rabbit IgG F(ab)'-FITC (Boehringer Mannheim) at 37° C for 1 hour. The slides were washed 5x with dilution buffer and sealed in 50% glycerol in PBS. Specific immunofluorescence was observed and micrographs were taken under NIKON™ epifluorescence microscope.

F. Western Blotting

Approximately 10^6 cells were directly dissolved in 100 ul loading buffer and heated to 95° C for 5 min. The sample was chilled on ice and loaded onto a minigel (Novex precasted SDS gel, 4-20%). The sample was fractionated with SDS electrophoresis at 75 mA until the dye front moved near the end. Electric transfer to nitrocellulose membrane was carried out in Tris-glysine-methanol buffer at 100 V for 1 hour. The membrane was washed with water and was saturated by incubation with 1% BA at 37° C with gentle shaking. Incubation of primary antibodies (anti-nestin antiserum 1:1000, anti-neuron specific antiserum 1:1000) was carried out at 37° C for 2 hours. Excess primary antibody were washed away with three changes of incubation buffer, 10 minutes for each change. The membrane was then incubated with anti-rabbit IgG F(ab)'-peroxidase for 1 hour. Finally, the membrane was stained with DAB-H_2O_2 as described above for immunocytochemistry.

Results

Neuroepithelial cells dissected from E9 rat embryos survived poorly in most serum free conditions. No cells survived beyond 4 days in conditions supplemented with EGF, FGFs, IGFs and neurotropins alone or in combination. Significant cell survival and proliferation was found only in those cells co-cultured with embryonic Schwann cells in the presence of Schwann cell growth medium which contained insulin, transferrin, α-tocopherol, progesterone, forskolin, bovine pituitary extract and recombinant human heregulin. Under these conditions, epithelial cells rapidly proliferated to form large cell colonies which pushed off the Schwann cells or grew underneath the Schwann cell monolayer. These cell colonies can form secondary colonies after subculture. Addition of bFGF to the secondary cultures induced the cells to extend long processes which morphologically resembled neuronal processes.

In order to achieve pure cells, embryonic Schwann cell conditioned medium (ESCCM) was tested, as well as cultures with trans-well inserts containing Schwann cells. This separates the Schwann cells from the neuroepithelial cells by a physical barrier but allows many Schwann cell secreted molecules to pass through. Both methods resulted in the survival and proliferation of a small proportion of neuroepithelial cells, which

formed large colonies of epithelial cells with some differentiated neurons which extended long processes after 10 days. No cells survived in conditions without ESCCM or coculture. The cells grown in the presence of ESCCM were successfully subcultured. During the first 5 to 6 passages, the cultures comprised a mixture of cell types, epithelial type of cells, spindle-shaped Schwann cell like cell type and aggregated small neurons with long and fine processes. Secondary cultures no longer require the Schwann cell conditioned medium. The epithelial type of cells were enriched and become dominant in subsequent cultures by removing heregulin a potent glial cell mitogen and keeping the cells at a high density.

These normal neural epithelial precursor cells required cell-cell contact for survival and growth. Dispersion of the culture to single cells, even with a brief trypsin treatment, resulted in cell death. Optimal growth was achieved when cells were plated at density of 1-2 x 10^5 cells/cm^2 A population doubling time of 50 hours was calculated, however the growth rate is non-linear with a marked increase in the doubling time as the cells get denser. Confluent culture reached a density 10^6 cells/cm^2 but always remained as a monolayer. The cells have been grown continuously for over 40 passages (>80 population doublings), no obvious change has been found in cell morphology and growth profile and no obvious cell senescence has been observed.

Cell morphology

The cell line showed monolayer epithelial cell morphology with a large nucleus/cytoplasm ratio during routine subculture as described. Most cells showed positive immunofluorescent staining for the neural precursor cell-specific intermediate filament protein, nestin. A western blot of solubilized cell membranes stained with anti-nestin antibody showed a single band at molecular weight 220 kilodaltons (Kd), consistent with the reported molecular weight of nestin. A small proportion (<5%)also showed positive staining for neuron specific enolase which had a molecular weight of 46 Kd in the western blot.

Growth factor response

The NEP cell line required the presence of insulin, bovine pituitary extract, and forskolin (or cholera toxin or cAMP analogs) for survival and growth. Removal of one of these three growth factors resulted in a sharp decrease in cell number (Figure 1A). Optimal concentrations of these growth factor were determined to be 10 ug/ml insulin, 3-10 uM forskolin and 0.3% (v/v) bovine pituitary extract (Figure 1B and 1C). Insulin can also be replaced by insulin-like growth factors. The cells are more sensitive to IGF-1 than to IGF-II (Figure 1D). Growth factors such as PDGF, EGF, heregulin, leukocyte inhibitory factor (LIF), hepatocyte growth factor (HGF) and neurotropins (NGF, NT-3 and BDNF) did not increase cell number (Figure 1E,F). Members of the TGFß family of growth factors had an inhibitory effect on cell growth (Figure 2A). Addition of TGFß1 resulted in significant cell death (Figure 2A). In the absence of forskolin and BPE, bFGF increased cell survival. While all the cells died in F12/DME supplemented with insulin only, with the addition of bFGF, a large proportion of cells survived as floating cell aggregates. However, addition of bFGF in the presence of 7F greatly inhibited cell proliferation, completely blocking cell growth beyond 24 hours of treatment. In contrast, aFGF showed little effect (Figure 2B).

In vitro differentiation of the NEP cell line:

To test this hypothesis, cells were grown in the presence of 6F alone (insulin, transferrin, bovine pituitary extract, heregulin, progesterone, and a-tocopherol), 6F + bFGF, or 6F + bFGF + forskolin, for 48 hours and then fixed and immunostained for a variety of neuronal markers. The cells showed a marked change in morphology in the presence of bFGF, and a further evolution towards neuronal- like morphology in the presence of bFGF + forskolin. These morphological changes were accompanied by the increased expression of neural specific markers visualized by immunohistochemistry. The number of cells expressing and the extent of expression of both vimentin and tubulin β was increased by FGF and further increased by the combination of bFGF and forskolin. Specific neuronal markers, such as Map-2, peripherin and the 160 kd neurofilament protein (NF 160) were also induced as the cells became more differentiated morphologically, although these were expressed to differing extents and in different ways under the different conditions. For example, NF 160 is expressed only in a few cell/cell junctions in the control conditions. With FGF alone there is continued expression at the junctions and some cytoplasmic expression while in the FGF + forskolin conditions NF 160 can be seen on the cell surface and in the neural extensions as well as cytoplasmically. While Map2 is expressed by most cells in the presence of both factors, peripherin is present on only a few of the cells. Cells exposed to both factors express protein kinase c (Pkc), as well as the excitatiry amino acids glutamate and aspartate. Neuron specific enolase, synaptophysin, and Tau were also induced in some cells in the cultures. Low levels of the glial markers such as GFAP and Gal-c were induced in some cells some conditions. These results are summarized in Table I.

Table I

Induction

Differentiation of neuroepithelial cell line			
antigens	control	bFGF 120 hr	forskolin+bFGF
Neuron specific Enolase	rare	30-50%	>90%
vimentin	rare	>90%	>90%
Tubulin-beta	rare	>90%	>90%
HNK-1	negative	negative	negative
GalC	negative	negative	negative
GFAP	negative	negative	very rare
P75-LNGFR	10-20%	10-20%	10-20%
Protein Kinase C (neuron specific)	some	>90%	>90%
Microtubule associated protein-2 (Map-2)	10-20%	30-50%	>90%
Neurofilament 68Kd	negative	10-20%	10-20%
Neurofilament 160Kd	junctional	cytoplasmic	cytoplasmic
Neurofilament 200Kd	negative	negative	3-5%
Choline Acetyl-transferase	negative	3-5%	3-5%
Tyrosine hydroxylase	negative	negative	negative
Serotonin	negative	negative	negative
GABA	negative	negative	negative
synaptophysin	negative	rare	90%
Tau	negative	negative	50%
periphorin	negative	rare	3-5%
glutamate	weak	weak	strong positive
aspartate	weak	weak	strong positive

EXAMPLE II

In vivo injection and differentiation of NEP cells

NEP cells were is lated and expanded as described above. Approximately 1 x 10^5 to 1 x 10^6 cells were removed from culture and resuspended in a balanced saline solution containing glucose. The cells were injected via a sterile microsyringe into neonatal rat brain.

NEP cells for implantation were labeled with the fluorescent viable cell marker, PKH-26. When injected into neonatal rat brain, the NEP cells gave rise to several distinct neural cell phenotypes. Two major cell types were identified with typical morphology within the appropriate layer of the cerebellum. These cells had the morphology characteristic of Bergman glia and granule cells. In the hippocampus, most of the fluorescently labeled cells integrated into the dentate girus. The majority of these cells had a morphology typical of dentate granule cells. Other cells showed neuronal-like differentiation but could not be precisely categorized by morphology. In the cerebral cortex, bundles of cell processes span the ventricular region up to the surface of the cortex with terminal branches which resemble radial glial cell processes. Some of the cell bodies were traced and were located inside the lateral ventricle. Integrated cells were also found in left hemisphere, although cells were injected only in the right hemisphere of the brain.

The majority of the neurons differentiated from NEP in vitro in the presence of bFGF and forskolin displayed markers consistent with a cortical neuron identity. The differentiated neurons expressed a protein kinase C isoenzyme which is expressed exclusively in CNS cortical neurons. In addition, these neurons accumulated the excitatory amino acids, glutamate and aspartate, in their cytoplasm and in cell processes. These amino acids are used as neurotransmitters only in cortical neurons. The in vitro data is confirmed by the results of the in vivo experiments.

In the presence of bFGF and forskolin, most NEP cells differentiated into aminonergic neurons. However, a small number of cells began to express choline acety-transferase, a key enzyme involved in acetyl-choline synthesis. The differentiation of cholinergic neurons in the NEP line was enhanced by addition of leukemia inhibiting factor (LIF). This result is consistent with the induction of cholinergic neurons by LIF in neural crest cells and hippocampal cells. Although no mature glial cells were identified in vitro, the NEP cells can differentiate into Bergmann glial cells when injected into the cerebellum and radial glial cells in the cerebral cortex. Taken together, these data suggest that the NEP cell line is arrested at an early stage of neuroepithelial differentiation and maintains the ability to differentiate into several different neuronal and glial cells depending on the environment.

EXAMPLE III

Isolation of human NEP cell line.

Neural tubes are obtained from, preferentially, embryos of 3-4 weeks of gestation. The dissected neural tubes are briefly treated with collagenase and dispase solution as described in the NEP culture described above. The cell aggregates are then washed free of the enzymes and plated on human laminin coated tissue culture dishes previously seeded with rat ESC or, preferably, human embryonic Schwann cells or Schwann cell conditioned medium. Culture medium for the culture of the human NEP cells is serum free medium containing human insulin, mammalian (preferably human) pituitary extract, forskolin, vitamin E., recombinant human heregulin, progesterone and transferrin. When large colonies of epithelial cells grow up cells can be subcultured as described (supra) for rat NEP cells. Schwann cells (if co-cultured) are removed by removing heregulin from the medium and differential enzyme digestion.

Deposit of Materials

The following culture has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

Cell Line	ATCC No.	Deposit Date
NEP95	CRL 11993	October 31, 1995

This deposit is being made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of deposit. The cell line will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture to the public upon issuance of the pertinent U.S. patent.

The assignee of the present application agrees that if the culture on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited cells are not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the culture deposited, since the deposited embodiments is intended as an illustration of an aspect of the invention and any cultures that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents.

WHAT IS CLAIMED IS:

1. A method of isolating a normal neural epithelial precursor cell type comprising the steps of:

a) culturing neural tissue from a mammalian embryo at the zero-somite stage of development in the presence of embryonic Schwann cells or embryonic Schwann cell conditioned media: and

b) isolating the normal neural epithelial precursor cells.

2. The method of claim 1 wherein the neural tissue is neural tube.

3. The method of claim 2 wherein the neural tissue is cultured on a solid support precoated with an attachment factor.

4. The method of claim 3 wherein the attachment factor is laminin.

5. The method of claim 4 wherein the neural tissue is cultured in serum free media.

6. The method of claim 5 wherein the serum free media comprises bovine pituitary extract, insulin, and forskolin.

7. The method of claim 6 wherein the serum free media further comprises progesterone, and α-tocopherol.

8. The method of claim 2 wherein the neural tube is derived from a rat embryo.

9. The method of claim 8 wherein the neural tube is from a day 9 embryo.

10. The method of claim 2 wherein the neural tube is derived from a human embryo.

11. The method according to claim 7 further comprising the step of separating the neural epithelial precursor cells from other cell types.

12. The method of claim 11 wherein the normal neural epithelial cells are subcultured in serum free medium lacking heregulin.

13. A normal neural epithelial precursor cell line isolated by the method of claim 1.

14. The normal neural epithelial precursor cell line of claim 13 which is a rat cell.

15. The normal neural epithelial precursor cell line of claim 13 which is a human cell.

16. The normal neural epithelial cell line of claim 14 which is on deposit with the American Type Culture Collection and bears Accession Number CRL 11993.

17. A composition comprising the cell line of claim 13 and a pharmaceutically acceptable vehicle.

18. A composition comprising the cell line of claim 14 and a pharmaceutically acceptable vehicle.

19. A composition comprising the cell line of claim 15 and a pharmaceutically acceptable vehicle.

20. A composition comprising the cell line of claim 16 and a pharmaceutically acceptable vehicle.

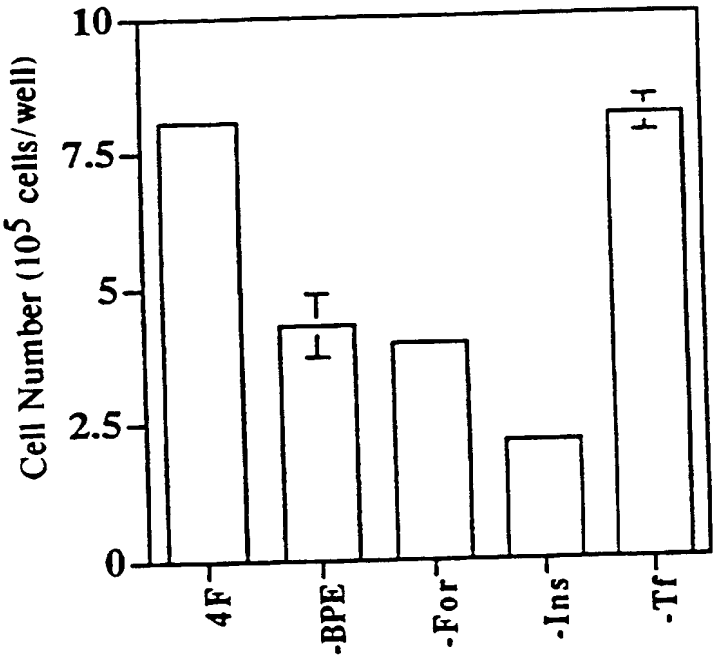

10
7.5
5
2.5
0
Cell Number (10^5 cells/well)
4F
-BPE
-For
-Ins
-Tf

FIG. 1A

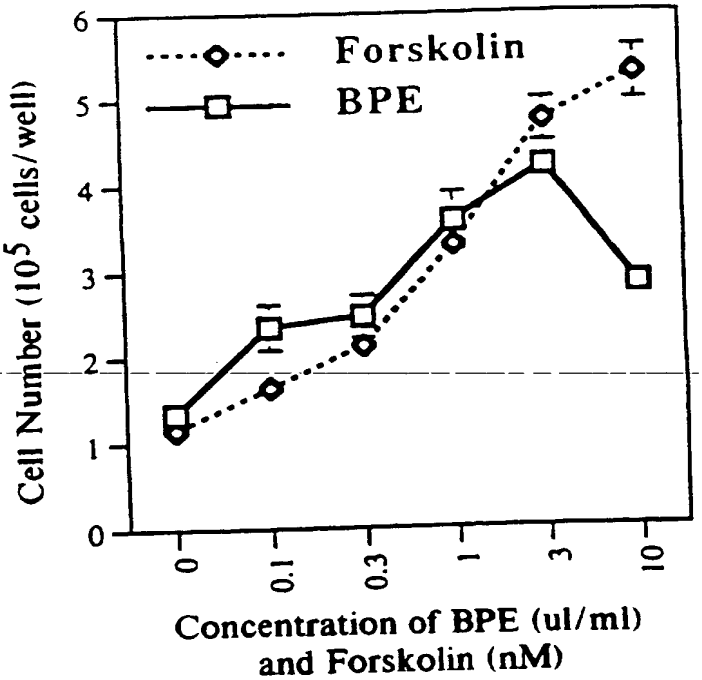

Forskolin
BPE
6
5
4
3
2
1
0
Cell Number (10^5 cells/well)
0
0.1
0.3
1
3
10
Concentration of BPE (ul/ml)
and Forskolin (nM)

FIG. 1B

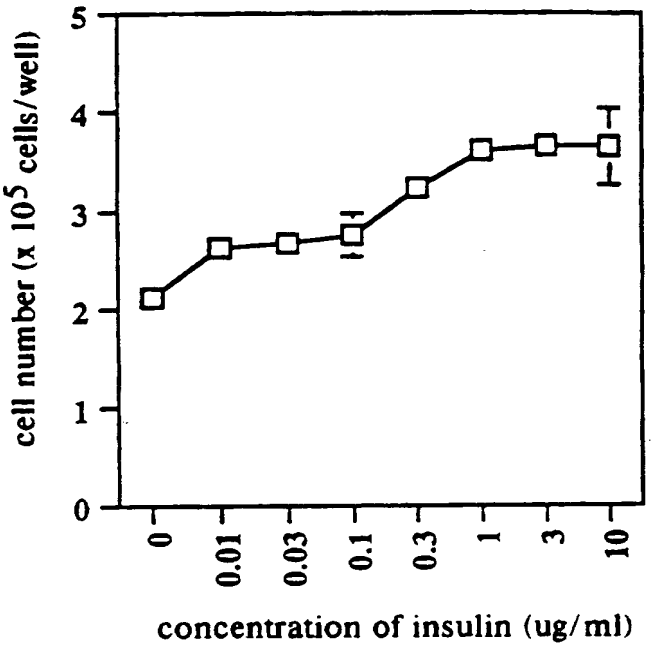


FIG. 1C

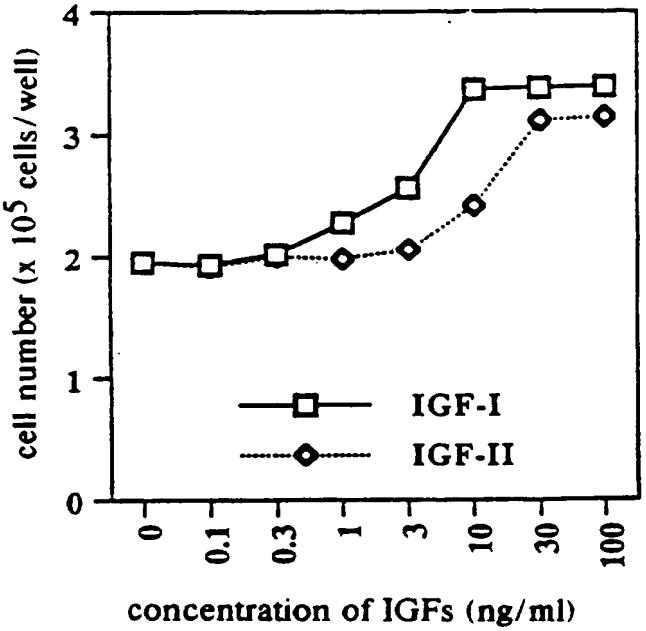


FIG. 1D

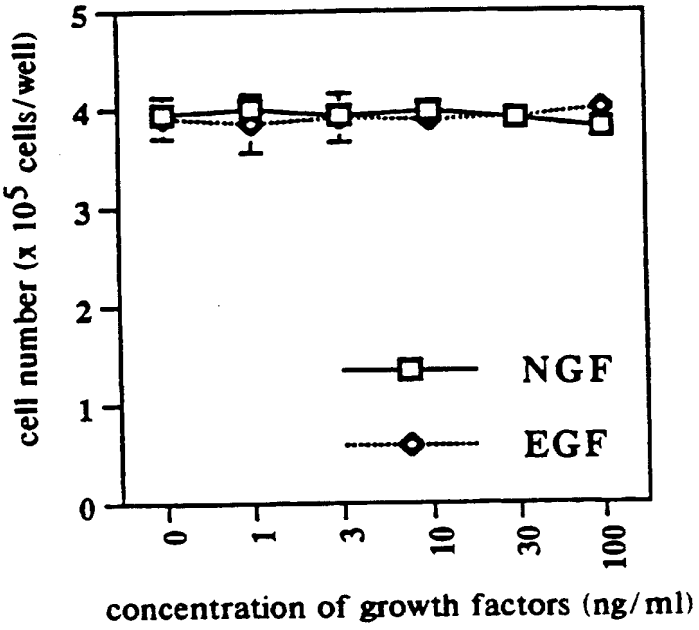


FIG. 1E

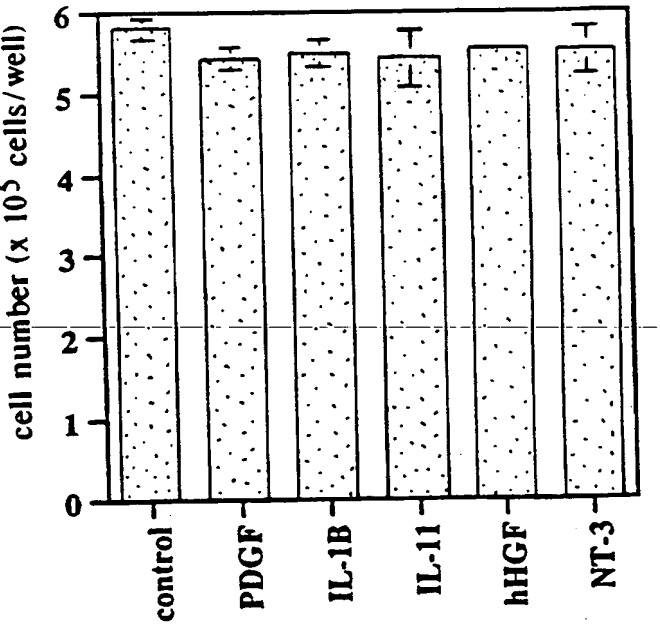


FIG. 1F

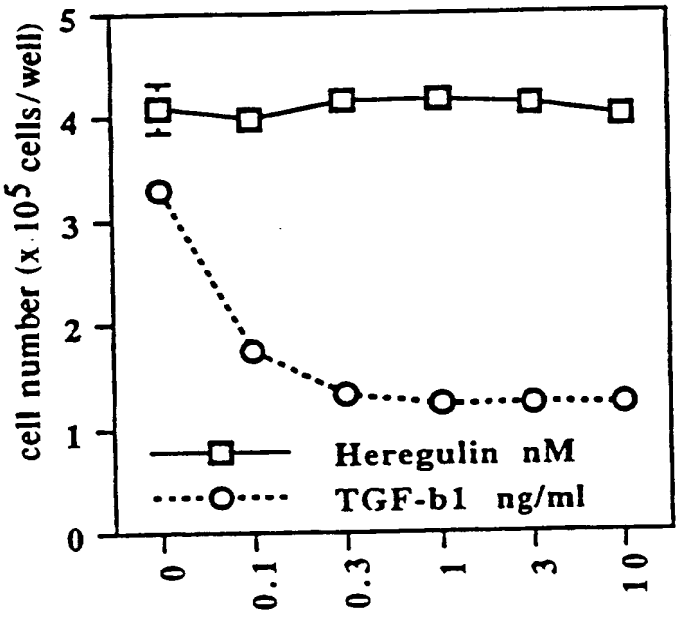


FIG. 2A

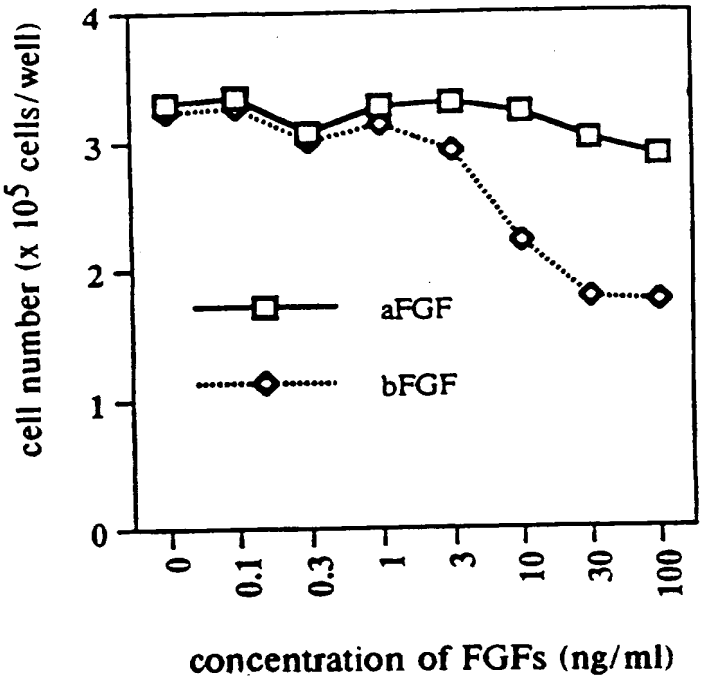


FIG. 2B

INTERNATIONAL SEARCH REPORT

al Application No
PCT/US 96/16543

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 C12N5/08 A61K35/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 02593 A (CALIFORNIA INSTITUTE OF TECHNOLOGY) 3 February 1994 see page 26, line 8 - page 28, line 6; claims; examples 1-6	1-20
P,X	ENDOCRINE, vol. 5, no. 2, October 1996, NEW YORK, N.Y., US, pages 205-217, XP000647380 R. LI ET AL.: "MULTIPLE FACTORS CONTROL THE PROLIFERTATION AND DIFFERENTIATION OF RAT EARLY EMBRYONIC (DAY 9) NEUROEPITHELIAL CELLS." see the whole document	1-20
E	WO 96 35776 A (GENENTECH) 14 November 1996 see page 27, line 17 - line 19	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 March 1997	26. 03. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/16543

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9402593 A	03-02-94	AU 4837593 A	14-02-94
		CA 2140884 A	03-02-94
		EP 0658194 A	21-06-95
		JP 8500245 T	16-01-96
		NZ 256154 A	24-02-97
		US 5589376 A	31-12-96
WO 9635776 A	14-11-96	AU 5578996 A	29-11-96

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 5/06, 5/10, 15/09, C12Q 1/00

A1

(11) International Publication Number: WO 95/12665

(43) International Publication Date: 11 May 1995 (11.05.95)

(21) International Application Number: PCT/US94/12647

(22) International Filing Date: 2 November 1994 (02.11.94)

(30) Priority Data:
08/145,175 3 November 1993 (03.11.93) US

(71) Applicant: DIACRIN, INC. [US/US]; Building 96, 13th Street, Charlestown Navy Yard, Charlestown, MA 02129 (US).

(72) Inventors: DINSMORE, Jonathan, H.; 28 White Place, Brookline, MA 02146 (US). RATLIFF, Judson; 22 Flint Avenue, Stoneham, MA 02180 (US).

(74) Agents: OLSTEIN, Elliot, M. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: EMBRYONIC STEM CELLS CAPABLE OF DIFFERENTIATING INTO DESIRED CELL LINES

(57) Abstract

An embryonic stem cell which may be induced to differentiate homogeneously into a desired primary cell line. The embryonic stem cell may be engineered with DNA, which encodes a protein or polypeptide which promotes differentiation of the stem cell into a specific cell line, such as, for example, a neuronal cell line, a muscle cell line, or a hematopoietic cell line. The DNA may encode a transcription factor found in the particular cell line. In another alternative, a desired cell line is produced from embryonic stem cells by culturing embryonic stem cells under conditions which provide for a three-dimensional network of embryonic stem cells, and then stimulating embryonic stem cells with an agent, such as retinoic acid, or dimethylsulfoxide, which promotes differentiation of the embryonic stem cells into the desired cell line, such as, for example, a neuronal cell line, or a muscle cell line.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

EMBRYONIC STEM CELLS CAPABLE OF DIFFERENTIATING INTO DESIRED CELL LINES

This application is a continuation-in-part of application Serial No. 08/145,175, filed November 3, 1993.

This invention relates to embryonic stem cells. More particularly, this invention relates to embryonic stem cells which are engineered with DNA and/or cultured in the presence of an agent, whereby such cells become capable of differentiating homogeneously into a desired primary cell line. Such homogeneous differentiation has not and cannot be achieved unless the methods described herein are applied.

Embryonic stem cells are pluripotent cells derived from the inner cell mass of pre-implantation embryos. (Evans et al., Nature, Vol. 292, pgs. 154-156 (1981)). Embryonic stem cells can differentiate into any cell type in vivo (Bradley, et al., Nature, Vol. 309, pgs. 255-256 (1984); Nagy, et al., Development, Vol. 110, pgs. 815-821 (1990) and into a more limited variety of cells in vitro (Doetschman, et al., J. Embryol. Exp. Morph., Vol. 87, pgs. 27-45 (1985); Wobus, et al., Biomed. Biochim. Acta, Vol. 47, pgs. 965-973 (1988); Robbins, et al., J. Biol. Chem., Vol. 265, pgs. 11905-11909 (1990); Schmitt, et al., Genes and Development, Vol. 5, pgs. 728-740 (1991)). Embryonic stem cells, however, are more difficult to maintain in the laboratory and require the

addition of a differentiation inhibitory factor (commonly referred to as leukemia inhibitory factor (or LIF) in the culture medium to prevent spontaneous differentiation (Williams, et al., Nature, Vol. 336, pgs. 684-687 (1988); Smith, et al., Nature, Vol. 336, pgs. 688-690 (1988); Gearing, et al,, Biotechnology, Vol. 7, pgs. 1157-1161 (1989); Pease, et al., Dev. Biol., Vol. 141, pgs. 344-352 (1990). LIF is a secreted protein and can be provided by maintaining embryonic stem cells on a feeder layer of cells that produce LIF (Evans, et al., 1981; Robertson, Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, Washington, D.C.: IRL Press (1987)) or by the addition of purified LIF (Williams, et al., 1988; Smith, et al., 1988; Gearing, et al., 1989; Pease, et al., Exp. Cell Res., Vol. 190, pgs. 209-211 (1990) to the medium in the absence of feeder layers. Differentiation of embryonic stem cells into a heterogeneous mixture of cells occurs spontaneously if LIF is removed, and can be induced further by manipulation of culture conditions (Doetschmann, et al., 1985; Wobus, et al., 1988; Robbins, et al., 1990; Schmitt, et al., 1991; Wiles, et al., Development, Vol. 111, pgs. 254-267 (1991); Gutierrez-Ramos, et al., Proc. Nat. Acad. Sci., Vol. 89, pgs. 9111-9175 (1992)). Differentiation of stem cells into a homogeneous population, however, has not been achieved. Embryonic stem cell differentiation can be variable between different established embryonic stem cell lines and even between laboratories using the same embryonic stem cell lines.

It is an object of the present invention to provide embryonic stem cells which are capable of differentiating uniformly into a specific and homogeneous cell line, not achievable by previous methods.

In accordance with an aspect of the present invention, there is provided a method of producing a desired cell line from embryonic stem cells. The method comprises culturing

embryonic stem cells under conditions which promote growth of the embryonic stem cells at an optimal growth rate. The embryonic stem cells then are cultured under conditions which promote the growth of the cells at a rate which is less than that of the optimal growth rate, and in the presence of an agent which promotes differentiation of the embryonic stem cells into the desired cell line.

In general, a growth rate which is less than the optimal growth rate, is a growth rate from about 10% to about 80%, preferably from about 20% to about 50%, of the maximum growth rate for embryonic stem cells. The growth rates for embryonic stem cells can be determined from the doubling times of the embryonic stem cells. In general, the optimum doubling time for embryonic stem cells is from about 13 hours to about 18 hours, and more particularly, from about 15 hours to about 16 hours.

In one embodiment, when the embryonic cells are being cultured under conditions which promote growth of the cells at an optimal growth rate, the embryonic stem cells are cultured in the presence of a medium including leukemia inhibitory factor (LIF), and serum selected from the group consisting of: (i) horse serum at a concentration of from about 5% by volume to about 30% by volume; and (ii) fetal bovine serum at a concentration of from about 15% by volume to about 30% by volume. In one embodiment, the serum is horse serum at a concentration of about 10% by volume. In another embodiment, the serum is fetal bovine serum at a concentration of about 15% by volume.

In yet another embodiment, when the embryonic stem cells are cultured at an optimal growth rate, the embryonic stem cells are cultured in the absence of a feeder layer of cells.

In one embodiment, the agent which promotes differentiation of the embryonic stem cells is selected from the group consisting of retinoic acid and nerve growth factor, and the desired cell line is a neuronal cell line.

In one embodiment, in addition to culturing the cells in the presence of the stimulating agent selected from the group consisting of retinoic acid and nerve growth factor, the embryonic stem cells are grown in the presence of a cytokine. Cytokines which may be employed include, but are not limited to, Interleukin-1, Interleukin-3, Interleukin-4, Interleukin-6, colony stimulating factors such as M-CSF, GM-CSF, and CSF-1, steel factor, and erythropoietin.

In a further embodiment, the agent which promotes differentiation of the embryonic stem cells is selected from the group consisting of dimethylsulfoxide and hexamethylene bis-acrylamide, and the desired cell line is a muscle cell line, such as a smooth muscle cell line, or a skeletal muscle cell line, or a cardiac muscle cell line. In one embodiment, the agent is dimethylsulfoxide. In another embodiment, the agent is hexamethylene bis-acrylamide.

In one embodiment, in addition to culturing the embryonic stem cells in the presence of an agent which promotes differentiation of the embryonic stem cells into a muscle cell line, the embryonic stem cells also are grown in the presence of a cytokine, examples of which are hereinabove described.

In yet another embodiment, when the embryonic stem cells are cultured in the presence of the agent which promotes differentiation of the embryonic stem cells into a desired cell line, the embryonic stem cells also are cultured in the presence of fetal bovine serum at a concentration of about 10% by volume.

In a further embodiment, when the embryonic stem cells are cultured in the presence of the agent which promotes differentiation of the embryonic cells into a desired cell line, the embryonic stem cells also are cultured in a three-dimensional format.

Thus, Applicants have found that one may produce a homogenous desired cell line from embryonic stem cells by

culturing the embryonic stem cells initially under conditions which favor the growth or proliferation of such embryonic stem cells at an optimal growth rate, and then culturing the cells under conditions which decrease the growth rate of the cells and promote differentiation of the cells to a desired cell type.

In a preferred embodiment, the embryonic stem cells cultured in a standard culture medium (such as, for example, Minimal Essential Medium), which may include supplements such as, for example, glutamine, and ß-mercaptoethanol. The medium may also include leukemia inhibitory factor (LIF), or factors with LIF activity, such as, for example, CNTF or IL-6, and horse serum. LIF, and factors with LIF activity, prevents spontaneous differentiation of the embryonic stem cells, and is removed prior to the addition of the agent. Horse serum promotes differentiation of the embryonic stem cells into the specific cell type after the addition of the agent to the medium. After the cells have been cultured for a period of time sufficient to permit the cells to proliferate to a desired number, the cells are washed free of LIF, and then cultured under conditions which provide for the growth of the cells at a decreased growth rate but which also promote differentiation of the cells. The cells are cultured in the presence of an agent which promotes or stimulates differentiation of the embryonic stem cells into a desired cell line, and in the presence of fetal bovine serum at a concentration of from about 5% by volume to about 10% by volume, preferably at about 10% by volume. The presence of the fetal bovine serum at a concentration of from about 5% by volume to about 10% by volume, and of the agent, provides for growth or proliferation of the cells at a rate which is less than the optimal rate, while favoring the differentiation of the cells into a homogeneous desired cell type. The desired cell type is dependent upon the agent which promotes or stimulates the differentiation of the embryonic stem cells.

The embryonic stem cells also are cultured in a three-dimensional format. Examples of such three-dimensional culturing formats are disclosed in Doetschman, et al., (1985), and in Rudnicki, et al., (1987).

For example, the embryonic stem cells may be placed in a culture vessel to which the cells do not adhere. Examples of non-adherent substrates include, but are not limited to, polystyrene and glass. The substrate may be untreated, or may be treated such that a negative charge is imparted to the cell culture surface. In addition, the cells may be plated in methylcellulose in culture media, or in normal culture media in hanging drops (Rudnicki, et al., 1987). Media which contains methylcellulose is viscous, and the embryonic stem cells cannot adhere to the dish. Instead, the cells remain isolated, and proliferate, and form aggregates.

In order to form aggregates in hanging drops of media, cells suspended in media are spotted onto the underside of a lid of a culture dish, and the lid then is placed on the culture vessel. The cells, due to gravity, collect on the undersurface of the drop and form aggregates.

In accordance with another aspect of the present invention, there is provided an embryonic stem cell. The embryonic stem cell has been engineered with DNA which encodes a protein or polypeptide which promotes differentiation of the cell into a specific cell line.

The DNA which encodes a protein or polypeptide which promotes differentiation of the embryonic stem cell into a specific cell line is DNA encoding a protein or polypeptide which is normally found in the specific differentiated cell line. Preferably, the protein or polypeptide which is present in the specific cell line is a protein or polypeptide which generally is not present in other types of cells.

In one embodiment, the DNA which encodes a protein or polypeptide which promotes differentiation of the embryonic stem cell into a specific differentiated cell line is DNA

encoding a transcription factor present in the specific cell line to promote differentiation of the cell into the specific cell line.

In one embodiment, the DNA encoding a transcription factor is DNA encoding a transcription factor present in neuronal cells, and the specific cell line is a neuronal cell line.

In another embodiment, the DNA encoding a transcription factor is DNA encoding a transcription factor, such as the MyoD gene, present in muscle cells, and the specific cell line is a muscle cell line.

In yet another embodiment, the DNA encoding a transcription factor is DNA encoding a transcription factor present in hematopoietic cells, and the specific cell line is a hematopoietic cell line.

The DNA which encodes a protein or polypeptide which promotes differentiation of the embryonic cell into a specific cell line may be isolated in accordance with standard genetic engineering techniques (such as, for example, by isolating such DNA from a cDNA library of the specific cell line) and placed into an appropriate expression vector, which then is transfected into embryonic stem cells.

Appropriate expression vectors are those which may be employed for transfecting DNA into eukaryotic cells. Such vectors include, but are not limited to, prokaryotic vectors such as, for example, bacterial vectors; eukaryotic vectors, such as, for example, yeast vectors and fungal vectors; and viral vectors, such as, but not limited to, adenoviral vectors, adeno-associated viral vectors, and retroviral vectors. Examples of retroviral vectors which may be employed include, but are not limited to, those derived from Moloney Murine Leukemia Virus, Moloney Murine Sarcoma Virus, and Rous Sarcoma Virus.

In a preferred embodiment, cDNA is synthesized from RNA isolated by the method of Chomczynski, et al., Anal.

Biochem., Vol. 162, pgs. 156-159 (1987) from cells of interest. All RNA preparations are screened for the presence of large RNAs with gene probes that recognize high molecular weight mRNA (i.e., greater than 6 kb) on Northern blots. For example, all RNA preparations from neural cells may be screened for detection of MAP2 mRNA on Northern blots. (MAP2 is a brain specific protein present in low abundance and coded for by a messenger RNA of about 9kb. The ability to detect MAP2 messenger RNA on a Northern blot is a stringent test for the presence of intact high quality RNA.)

For cDNA synthesis, a single tube method developed by Gubler, Nucl. Acids Res., Vol. 16, pg. 2726 (1988) is employed, and conditions are optimized to yield the greatest amount of full length cDNA product (about 7.5 kb in length). The cDNA is inserted into the pcDNA3 vector (Invitrogen), which allows for expression of the cDNA insert in mammalian cells. The pcDNA3 vector contains the cytomegalovirus (CMV) promoter, the SV40 origin of replication, the neomycin resistance gene for selection in eukaryotic cells, and the ampicillin resistance gene for selection in bacteria such as E.coli.

cDNA libraries are constructed wherein all the clones are oriented in the proper orientation for expression. Such is achieved by synthesizing oligo (dT) primed libraries with an oligo (dT) primer that includes a NotI site, and after cDNA synthesis, a BstXI adapter is ligated to the cDNA. Finally, the cDNA is digested with NotI (an enzyme that cuts infrequently in eukaryotic genes), thus creating a cDNA with a NotI overhang at the 3' end and a BstXI overhang at the 5' end. The cDNA then is ligated into pcDNA3 digested with BstXI and NotI. This places the 5' end of the cDNA downstream from the CMV promoter.

To enrich for developmentally expressed genes, libraries from uninduced embryonic stem cells are screened with labeled cDNA from differentiated embryonic stem cells and all cross-

hybridizing clones are eliminated from further analysis. Such method allows the removal of those elements common to differentiated and undifferentiated cells. Also, subtractive cDNA libraries are constructed according to the method of Sive, et als., Nucl. Acids Res., Vol. 16, pg. 10937 (1988). Subtractive cDNA libraries are cDNA libraries that are enriched for genes expressed in one cell type but not in another. The method relies on removal of common DNA sequences through hybridization of similar DNA sequences, and then the removal of these hybridized double-stranded DNAs. A subtractive cDNA library that contains sequences specific for a particular cell type derived from induced embryonic stem cells is generated. Single stranded cDNA is synthesized from uninduced cells. To select for those genes that are specific for the desired cell line derived from embryonic stem cells, genes that are expressed both in the induced cells and the non-induced embryonic stem cells are removed. Thus, RNA which is isolated and purified from embryonic stem cells that have differentiated into a desired cell line is hybridized to an excess of cDNA synthesized from uninduced embryonic stem cells to insure that all common elements are removed. RNA and cDNA common to both the induced and uninduced embryonic stem cells will hybridize, and these hybrids are removed. To remove double-stranded material, cDNA from uninduced embryonic stem cells is covalently modified with photoactivatable biotin (Sive, et al., 1988), and the hybrid can be removed by a simple phenol extraction because the biotin on the cDNA will cause the hybrid to partition to the phenol phase while the non-hybridized RNA will partition to the aqueous phase. Following this selection, RNA species found specifically in differentiated embryonic stem cells are used to construct cDNA libraries as hereinabove described.

Plasmid DNA containing cDNA inserts then are electroporated into embryonic stem cells. Cells are

transfected with a plasmid that contains sequences for neomycin resistance and stable transfectants are isolated based on neomycin resistance. Stable transfected clones are isolated and induced with an appropriate agent, or with leukemia inhibitory factor (LIF) withdrawal alone, and scored for an increased ability to differentiate in response to these induction signals. Clones also are examined to determine if they are differentiating spontaneously in the presence of LIF.

In accordance with another aspect of the present invention, there is provided a method of producing a desired cell line from embryonic stem cells. The method comprises engineering embryonic stem cells with DNA which encodes a protein or polypeptide which promotes differentiation of the embryonic stem cells into a specific cell line. The embryonic stem cells then are stimulated with an agent which promotes differentiation of the embryonic stem cells into the desired cell line.

In one embodiment, the DNA which encodes a protein or polypeptide which promotes differentiation of the embryonic stem cells into a specific cell line is DNA encoding a transcription factor present in neuronal cells and said agent is selected from the group consisting of retinoic acid and nerve growth factor. In one alternative, the cells also may be grown in the presence of a cytokine such as those hereinabove described.

In another embodiment, the DNA which encodes a protein or polypeptide which promotes differentiation of the embryonic stem cells into a specific cell line is DNA encoding a transcription factor, such as, for example, the MyoD gene, present in muscle cells and said agent is a bipolar agent such as dimethylsulfoxide or hexamethylene bis-acrylamide. In one alternative, the embryonic stem cells also may be grown in the presence of a cytokine.

The embryonic stem cells may be engineered with the DNA and cultured under conditions hereinabove described. For example, prior to induction, the embryonic stem cells are engineered with DNA which encodes a protein or polypeptide which promotes differentiation of the embryonic stem cells into a specific cell line. Then, the embryonic stem cells may be cultured under conditions which provide for a three-dimensional network of such cells.

Also, it is to be understood that, within the scope of the present invention, that the embryonic stem cells may be used for gene therapy purposes. The embryonic stem cells may be engineered with DNA encoding a desired therapeutic agent. Such engineering may be accomplished by using expression vectors such as those hereinabove described. Once the cells are engineered with DNA encoding a desired therapeutic agent, the cells then are engineered with DNA which encodes a protein or polypeptide which promotes differentiation of the embryonic stem cells into a specific desired cell line, and/or stimulated with an agent which promotes differentiation of the embryonic stem cells into a desired cell line. The differentiated cells then may be administered to a host, such as a human or non-human host, as part of a gene therapy procedure.

In addition, there is also provided within the scope of the present invention, a method of screening embryonic stem cells for proteins which induce differentiation of embryonic stem cells into desired cell lines. In such method, RNA is obtained from specifically desired cells or tissues (such as for example, brain cells), and cDNA libraries are then constructed and placed into expression vectors. The libraries may be normal cDNA libraries or they may be subtractive cDNA libraries, i.e., such DNA libraries include DNA found in the desired cells or tissues but not in other cells or tissues. The expression vectors are then transfected into eukaryotic cells, such as COS cells. The

cell culture supernatant then may be applied to embryonic stem cell cultures to determine if any secreted proteins from such cells induce differentiation of embryonic stem cells to a specific cell type. The cDNA from cells which induce differentiation of embryonic stem cells to a specific cell type then is evaluated further in order to determine which individual clones of such cDNA libraries induce differentiation of embryonic stem cells to a specific cell type. Once a specific cDNA which induces differentiation of embryonic stem cells to a desired cell type is identified, such cDNA then may be isolated and cloned into an appropriate expression vector, which may be transfected into undifferentiated embryonic stem cells or the expressed, purified protein may be added directly to cultured embryonic stem cells.

In one embodiment, such screening may be carried out by pooling bacterial clones, from the cDNA library prepared as hereinabove described, into groups of 1,000, and isolating plasmid DNA from the pooled clones. The plasmid DNA's then are electroporated into COS cells, such as COS-7 cells, for expression. After allowing from 48 to 72 hours for expression of transfected genes, tissue culture supernatant from transfected COS cells is harvested and applied to embryonic stem cells to determine if any secreted proteins from the COS cells can induce differentiation of embryonic stem cells. Supernatants from mock transfected cells (cells transfected with the plasmid alone) are tested in parallel to control for any non-specific effects of COS cell derived proteins. Embryonic stem cell differentiation may be screened by several means: (i) by microscopic observation of overt changes in embryonic stem cell morphology; (ii) by measuring changes in neuron specific gene expression on Northern blots with probes to neuron specific markers such as neuron specific enolase, GAP-43, and MAP2; and (iii) by loss of expression of a carbohydrate surface marker present only

on undifferentiated stem cells recognized by the monoclonal antibody SSEA-1 (Ozawa, et al., Cell. Diff., Vol. 16, pp. 169-173 (1985)).

When a pool has been identified that expresses inducing capacity, that pool of cDNA clones is broken down further into smaller pools of 100 clones, and these sub-pools are transfected into COS cells. Supernatants are screened for inducing activity. Once appropriate sub-pools are identified, the clones are plated in 96 well dishes, and rows and columns are combined. The pooled columns and rows then are transfected into COS cells, and supernatants again are screened for activity. By analyzing the columns and rows that exhibit activity, the exact clone expressing inducing activity can be identified. This clone then is tested for ability to induce differentiation. After initial identification of potential factors, full-length cDNA clones are isolated and sequenced. Sequenced clones then are compared to other cloned genes in the DNA data base for homology or identity with previously cloned genes. Once a novel gene is identified, the gene is cloned into a stable expression system, the protein is purified, and its biological activity is tested. Sequencing of DNA is performed by standard protocols. Biologically active protein is prepared by standard chromatographic methods.

Alternatively, cDNA from differentiating embryonic stem cells or from embryonic organs and brain regions can be introduced directly into embryonic stem cells, and embryonic stem cell supernatants are screened for inducing activity.

The differentiated stem cells may be employed by means known to those skilled in the art to treat a variety of diseases or injuries. For example, stem cells which have differentiated into neuronal cells may be administered to a patient, such as, for example, by transplanting such cells into a patient, to treat diseases such as Huntington's disease, Parkinson's disease, and Alzheimer's disease. Such

neuronal cells also may be employed to treat spinal cord injuries or chronic pain. Also, stem cells which have differentiated into muscle cells may be employed in treating muscular dystrophy, cardiomyopathy, congestive heart failure, and myocardial infarction, for example.

The invention will now be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

Example 1

Undifferentiated embryonic stem cells (ES-E14TG2a, purchased from the American Type Culture Collection, catalog no. ATCC CRL 1821) are maintained in Dulbecco's modified Minimal Essential Medium (DMEM) supplemented with glutamine, ß-mercaptoethanol, 10% (by volume) horse serum, and human recombinant Leukemia Inhibitory Factor (LIF). The LIF replaces the need for maintaining embryonic stem cells on feeder layers of cells, and is essential for maintaining embryonic stem cells in an undifferentiated state.

In order to promote the differentiation of the embryonic stem cells into neuronal cells, the embryonic stem cells are trypsinized and washed free of LIF, and placed in DMEM supplemented with 10% (by volume) fetal bovine serum (FBS). After resuspension in DMEM and 10% FBS, $1X10^6$ cells are plated in 5ml DMEM plus 10% FBS plus 0.5µM retinoic acid in a 60mm Fisher brand bacteriological grade Petri dish. In such Petri dishes, embryonic stem cells cannot adhere to the dish, and instead adhere to each other, thus forming small aggregates of cells. Aggregation of cells aids in enabling proper cell differentiation. After two days, aggregates of cells are collected and resuspended in fresh DMEM plus 10% FBS plus 0.5µM retinoic acid, and replated in Petri dishes for an additional two days. Aggregates, now induced four days with retinoic acid, are trypsinized to form a single-cell suspension, and plated in medium on poly-D-lysine-coated

tissue culture grade dishes. The stem cell medium is formulated with Kaighn's modified Ham's F12 as the basal medium with the following supplements added:

15μg/ml ascorbic acid
0.25% (by volume) calf serum
6.25μg/ml insulin
6.25μg/ml transferrin
6.25ng/ml selenous acid
5.35μg/ml linoleic acid
30pg/ml thyroxine (T3)
3.7ng/ml hydrocortisone
1.ng/ml Heparin 10ng/ml somatostatin
10ng/ml Gly-His-Lys (liver cell growth factor)
0.1μg/ml epidermal growth factor (EGF)
50μg/ml bovine pituitary extract (BPE)

Such medium provides for consistent differentiation of the stem cells into neuronal cells, and provides for survival of the neuronal cells for a period of time greater than 3 days, and selectively removes dividing non-neuronal cells from the population. The poly-D-lysine promotes the attachment of the neuronal cells to the tissue culture plastic, and prevents detachment of the cells from the dish and the forming of floating aggregates of cells. The cells are cultured for 5 days. Upon culturing of the cells in the above medium, a culture of cells in which greater than 90% of the cells are neuronal cells is obtained. Such neuronal cells, which express the neurotransmitter gamma amino butyric acid (GABA), then may be employed for the treatment of the neural degeneration disease Huntington's disease. Through genetic engineering, these cells can be directed to express dopamine (for the treatment of Parkinson's disease) or acetylcholine (for the treatment of Alzheimer's disease).

Example 2

Undifferentiated embryonic stem cells (ES-D3, purchased from the American Type Culture Collection as ATCC catalog no. ATCC CRL 1934) are maintained in supplemented Dulbecco's modified Minimal Essential Medium as described in Example 1. The embryonic stem cells then are trypsinized and washed free of LIF and placed in 1% (by volume) dimethylsulfoxide in DMEM plus 10% horse serum. Two days after the addition of dimethylsulfoxide and plating of cells in Petri dishes to form aggregates, the aggregates are collected and resuspended in fresh medium plus 1% dimethylsulfoxide. The aggregates are then plated onto multi-well untreated culture grade dishes without trypsin treatment. One aggregate is plated per well. The aggregates are cultured for 5 days. Upon culturing of the cells in multi-well dishes, cell cultures in which greater than 80% of the aggregates contain contracting muscle cells are obtained. Such cells may be used to treat cardiomyopathies, myocardial infarction, congestive heart failure, or muscular dystrophy.

Example 3

Transfection of embryonic stem cells with mouse MyoD cDNA

For transfection of embryonic stem cells with mouse MyoD cDNA, both the D3 (ATCC catalog no. CRL 1934) and E14 TG2a (ATCC catalog no. CRL 1821) embryonic stem cell lines were used. Embryonic stem cells were cultured as described in Robertson, 1987, except that the cells were maintained in media containing 5 ng/ml human recombinant leukemia inhibitory factor instead of on feeder layers.

Embryonic stem cells were co-transfected with pKJ1-Neo (Dinsmore, et al., Cell, Vol. 64, pgs. 817-826 (1991)), which carries the neomycin resistance gene for selection of stable transfectants, and with pEMCII (Davis, et al., Cell, Vol. 51, pgs. 987-1000 (1987)), which contains a portion of the mouse

MyoD cDNA. pKJ1-Neo was linearized at the unique NsiI site and pEMCII was linearized at the unique ScaI site. In order to introduce the linearized plasmids into the embryonic stem cells, the embryonic stem cells were electroporated using a Gene Pulser (Bio Rad) in 0.4 cm gap distance electroporation cuvettes with the Gene Pulser set at 240 volts, 500μ Farads. For electroporation, $8x10^6$ embryonic stem cells were suspended in 1 ml of HEPES-buffered saline (25mM HEPES, 134mM Na Cl, 5mM KCl, 0.7mM Na_2 HPO_4, pH 7.1) with 2 μg of linearized pKJ1-Neo and 20μg pEMCII. After electroporation, the cells were plated at $5-7x10^5$ per 35mm gelatin coated culture dish in growth medium containing recombinant human leukemia inhibitory factor. The cells were allowed to grow for 36 hours and then Geneticin (Gibco-BRL), a commercial brand of neomycin, was added to the medium at a concentration of 400μg/ml. The media containing the Geneticin was changed daily until clones of neomycin resistant cells could be identified (7 days after Geneticin addition). Individual neomycin resistant clones were isolated using glass cloning cylinders (Bellco).

Stable transfectants were isolated, expanded, frozen, and then stored in liquid nitrogen. 35 independent stably transfected embryonic stem cell lines were isolated. Ten of these cell lines have been analyzed, and have been found to express different amounts of MyoD as detected by Northern blots. Embryonic stem cell lines that were found to express high levels of MyoD RNA were found to have embryonic stem cells in the population that spontaneously differentiated into muscle cells as assessed by the staining of cells with a muscle specific myosin antibody. Those cell lines which showed high levels of MyoD expression were characterized further by inducing differentiation with dimethylsulfoxide. Cell lines which expressed high amounts of MyoD differentiated almost exclusively into skeletal muscle after dimethylsulfoxide induction. The percentage of cells that

differentiated into skeletal muscle was greater than 90% as assessed by staining for muscle specific myosin, and by the ability of these cells to fuse and form myotubes that spontaneously twitch. In contrast, MyoD transformants that expressed very low amounts of MyoD differentiated into a mix of cardiac, smooth, and skeletal muscle indistinguishable from that derived from non-transfected embryonic stem cells. Additionally, there was no detectable difference between the D3 and E14 embryonic stem cell lines for MyoD expression or differentiation.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. A method of producing a desired cell line from embryonic stem cells, comprising:

— (a) culturing embryonic stem cells under conditions which promote growth of said cells at an optimal growth rate; and

(b) culturing said embryonic stem cells under conditions which promote growth of said cells at a rate which is less than that of said optimal growth rate, and in the presence of an agent which promotes differentiation of said embryonic stem cells into said desired cell line.

2. The method of Claim 1 wherein, in step (a), said embryonic stem cells are cultured in the presence of a medium including leukemia inhibitory factor, CNTF, or IL-6; and serum selected from the group consisting of (i) horse serum at a concentration of from about 5% by volume to about 30% by volume; and (ii) fetal bovine serum at a concentration of from about 15% by volume to about 30% by volume.

3. The method of Claim 2 wherein, in step (a), said serum is horse serum at a concentration of about 10% by volume.

4. The method of Claim 2 wherein, in step (a), said serum is fetal bovine serum at a concentration of about 15% by volume.

5. The method of Claim 1 wherein, in step (a), said embryonic stem cells are cultured in the absence of a feeder layer of cells.

6. The method of Claim 1 wherein, in step (b), said agent is selected from the group consisting of retinoic acid and nerve growth factor, and said desired cell line is a neuronal cell line.

7. The method of Claim 6 wherein said agent is retinoic acid.

8. The method of Claim 7 wherein, in addition to culturing said embryonic stem cells in the presence of said

retinoic acid, said embryonic stem cells are grown in the presence of a cytokine.

9. The method of Claim 1 wherein, in step (b), said embryonic stem cells are cultured in the presence of fetal bovine serum at a concentration of about 10% by volume.

10. Neuronal cells produced by the method of Claim 6.

11. The method of Claim 1 wherein, in step (b), said agent is selected from the group consisting of dimethylsulfoxide and hexamethylene bis-acrylamide, and said desired cell line is a muscle cell line.

12. The method of Claim 11 wherein said agent is dimethylsulfoxide.

13. The method of Claim 11 wherein said agent is hexamethylene bis-acrylamide.

14. The method of Claim 11 wherein, in addition to culturing said embryonic stem cells in the presence of an agent selected from the group consisting of dimethylsulfoxide and hexamethylene bis-acrylamide, said embryonic stem cells are grown in the presence of a cytokine.

15. Muscle cells produced by the method of Claim 11.

16. An embryonic stem cell, said embryonic stem cell having been engineered with DNA which encodes a protein or polypeptide which promotes differentiation of said cell into a specific cell line.

17. The cell of Claim 16 wherein said DNA which encodes a protein or polypeptide which promotes differentiation of said cell into a specific cell line is DNA encoding a transcription factor present in said specific cell line to promote differentiation of said cell into said specific cell line.

18. The cell of Claim 17 wherein said DNA encoding a transcription factor is DNA encoding a transcription factor present in neuronal cells, and said specific cell line is a neuronal cell line.

19. The cell of Claim 17 wherein said DNA encoding a transcription factor present in a specific cell line is DNA encoding a transcription factor present in muscle cells, and said specific cell line is a muscle cell line.

20. The cell of Claim 17 wherein said DNA encoding a transcription factor is DNA encoding a transcription factor present in hematopoietic cells, and the specific cell line is a hematopoietic cell line.

21. Neuronal cells produced from the cell of Claim 18.

22. Muscle cells produced from the cell of Claim 19.

23. Hematopoietic cells produced from the cell of Claim 20.

24. A method of producing a desired cell line from embryonic stem cells, comprising:

engineering said embryonic stem cells with DNA which encodes a protein or polypeptide which promotes differentiation of said embryonic stem cells into a specific cell line; and

stimulating said embryonic stem cells with an agent which promotes differentiation of said embryonic stem cells into said desired cell line.

25. The method of Claim 24 wherein said DNA which encodes a protein or polypeptide which promotes differentiation of said embryonic stem cells into a specific cell line is DNA encoding a transcription factor present in neuronal cells and said agent is retinoic acid.

26. Neuronal cells produced by the method of Claim 25.

27. The method of Claim 24 wherein said DNA which encodes a protein or polypeptide which promotes differentiation of said embryonic stem cells into a specific cell line is DNA encoding a transcription factor present in muscle cells and said agent is selected from the group consisting of dimethylsulfoxide and hexamethylene bis-acrylamide.

28. Muscle cells produced by the method of Claim 27.

29. A method of screening embryonic stem cells for proteins which induce differentiation of embryonic stem cells into a desired cell line comprising:

(a) obtaining RNA from a desired cell or tissue;

(b) constructing cDNA libraries from said RNA obtained from said desired cell or tissue;

(c) cloning such cDNA libraries into expression vectors;

(d) transfecting said vectors into eukaryotic cells;

(e) contacting embryonic stem cells with culture supernatant from said transfected eukaryotic cells to determine if proteins contained in said culture supernatant induce differentiation of said embryonic stem cells into a desired cell line;

(f) obtaining cDNA clones from those cells which induce differentiation of embryonic stem cells into a desired cell line;

(g) transfecting said cDNA clones from said cells which induce differentiation of embryonic stem cells into a desired cell line into eukaryotic cells;

(h) contacting embryonic stem cells with culture supernatant obtained from the eukaryotic cells of step (g);

(i) obtaining cDNA clones from those cells which induce differentiation of embryonic stem cells into a desired cell line;

(j) transfecting said cDNA clones from step (i) into embryonic stem cells; and

(k) determining at least one cDNA clone which induces differentiation of embryonic stem cells into a desired cell line.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12647

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/6, 172.1, 240.1, 240.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 172.1, 240.1, 240.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Medline, WPI
Search terms: embryonic stem cells, differentiation, genetic engineering, retinoic acid, cytokine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 51, issued 24 December 1987, Davis et al, "Expression of a Single Transfected cDNA Converts Fibroblasts to Myoblasts", pages 987-1000, see pages 987-994.	16-29
Y	WO, A, 90/03432 (EVANS ET AL.) 05 April 1990, see pages 11-15 and 20.	1-29
Y	Journals of Reproduction & Fertility, Volume 41, issued 1990, Notarianni et al, "Maintenance and Differentiation in Culture of Pluripotential Embryonic Cell Lines from Pig Blastocysts", pages 51-56, see pages 52-55.	1-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
02 FEBRUARY 1995

Date of mailing of the international search report
13 FEB 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
Sally P. Teng
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12647

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Neuroscience Research, Volume 12, issued 1992, Yamamori, "Molecular Mechanisms for Generation of Neural Diversity and Specificity: Roles of Polypeptide Factors in Development of Postmitotic Neurons", pages 545-582, see pages 545, 548, 554, and 566.	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12647

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 5/06, 5/10, 15/09; C12Q 1/00